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Pathogenesis of Mouse Viral Leukemia.* (26368)

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Virus-induced thymic lymphoma, with secondary generalized leukemia, is anatomically identical with the spontaneous disease in the high leukemia AK strain. The striking difference between them is the considerably shorter latency of virus-induced disease.

A study of the pathogenesis of viral-induced leukemia was undertaken by sacrificing mice at various intervals after viral infection, examining the organs, notably the thymus, for leukemic changes and assaying the thymus for malignant lymphocytes by grafting thymic fragments on young, highly susceptible, isologous mice.

* Supported by NCI Grant.

The site and origin of the leukemic cell of spontaneous thymic lymphoma in the AK strain was determined 16 years ago(1) by a similar procedure. The observations on viral-induced leukemia, here reported, suggest the essential identity of its pathogenesis with that of spontaneous leukemia, the only difference being shorter latency for neoplastic transformation of thymic lymphocytes following viral infection at neonatal age.

Materials and methods. Virus. Four different filtrates of Gross's passage virus A(2), which has been carried in our laboratory since 1957, were used. Preparation and storage of filtrates have been described(3).

Mice. AK/Z mice (F_1 hybrid from AKR/Jax females and C3H/f/Bi/G/L[1] males) were used. Newborn litters (less than one week of age) were injected subcutaneously with 0.025 to 0.05 cc of filtrate. Alternate control litters were either not injected or injected subcutaneously with 0.05 cc of phosphate buffer at less than one day of age. Littermates (males and females) were caged together; offspring of virus-injected females were discarded. *Intercurrent infections* were treated by addition of veterinary terramycin to drinking water.

Experimental procedures. Some virus-injected and control mice were observed until natural death; others were sacrificed at regular intervals. Sections were taken of numerous organs; special attention was given to thymus. Kidney and thyroid were routinely searched for microscopic polyoma lesions. Sections of virus-injected and control mice were studied simultaneously, as unknowns.

Transplantation assays were performed with thymuses taken at various intervals by grafts made intramuscularly in thighs of isologous mice. When only one recipient was used, one-half of each lobe of thymus was grafted in both thighs; when 2 recipients were used, one-half of each lobe was grafted on each recipient; the other halves of each thymic lobe were processed for microscopic study. When 4 recipients were used, about one-third of each lobe was processed for section; the remaining two-thirds of each lobe was cut into 4 equal pieces, and each recipient was grafted with one piece of each lobe in each thigh.

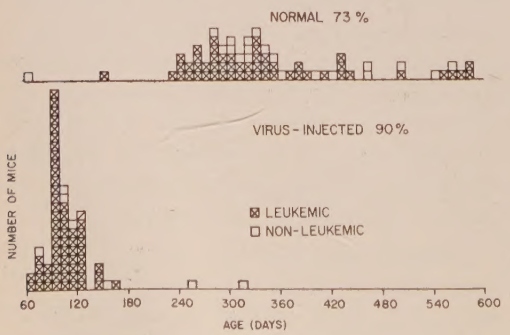


FIG. 1. Incidence and time of occurrence of leukemia in virus-inj. and normal uninj. AK/Z mice.

TABLE I. Microscopic Findings in Thymuses of Virus-Injected AK/Z Mice.

Age of thymus (days)	No. studied	Microscopic findings*
13-20	6	N N N N N N
21-28	7	±? N N N N N N
29-35	8	±? N N N N N atr atr
36-42	14	±? N N N N N N N N N N N N atr
43-49	6	±? N? N? N N atr
50-56	10	+ + N N N N N N N N
57-63	7	+ + ±? N? N? N N
64-70	5	+ + ±? ±? atr
71-77	9	+ + + + + + + + +

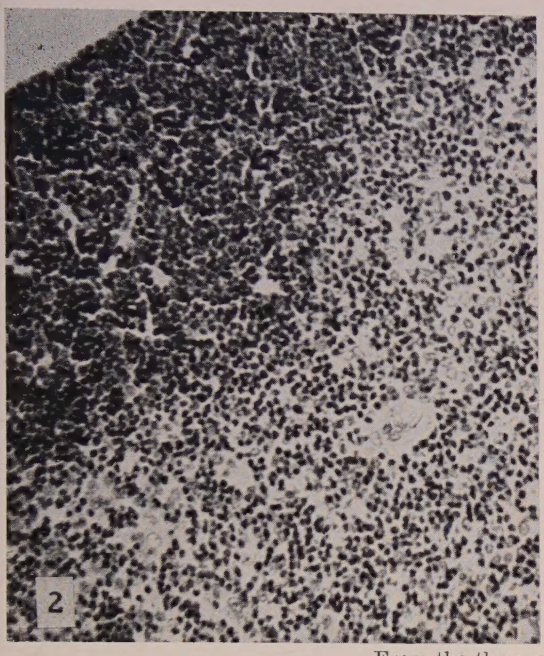
* Each letter or symbol refers to one mouse; N = normal, ±? = questionable preleukemic change, + = distinct leukemic change, atr = atrophy, N? = probably normal.

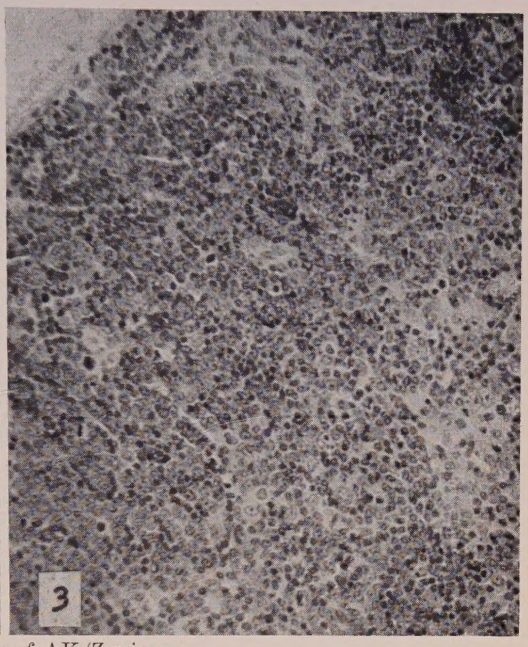
Results. Incidence and time of occurrence of leukemia in 67 virus-injected and 73 control mice are shown in Fig. 1. Virus-injected mice had a higher incidence of leukemia (90%) than normal mice (73%). However, leukemia occurred much earlier in virus-injected than in control mice. The latency period was slightly shorter in females than in males in both groups, as has been noted in spontaneous leukemias of several strains.

Morphogenesis (Table I). During the first few days of life the cortical area of the thymus of both injected and control mice was wide containing many immature medium to large lymphocytes; the medulla was wide with abundant reticular-epithelial cells. Maturation to the adult pattern, in which the predominant cell of the deeply-staining cortex is the small lymphocyte, was not retarded by virus.

Earliest morphologic change in virus-injected mice was noted at about 40 days, becoming more definite after 50 days. At this time, disseminated small foci of medium to large lymphocytes appeared either subcortically or somewhat deeper in the thymus. Subsequently, these areas enlarged, became confluent (Fig. 3, 5 *vs.* Fig. 2, 4), and by 70 days began to spread beyond the capsule. This sequence of events occurred with remarkable uniformity.

The thymus of 5 animals was involuted without any evidence of leukemia. These in-





From the thymus of AK/Z mice

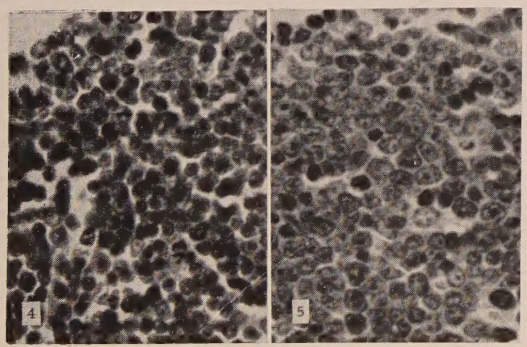
FIG. 2. Uninj., 45-day-old mouse. × 200.
FIG. 3. Virus-inj., 62-day-old mouse. Bio-assay positive. × 200.

voluted thymuses were similar to those of uninjected animals and were presumably due to some stress other than viral infection.

Transplantation assays of thymuses from virus-injected animals were performed for presence of neoplastic cells. Results corroborated the microscopic findings as shown in Table II. These assays failed to disclose the presence of neoplastic cells before 57 days of age, with 3 probable exceptions.

One 35-day-old thymus produced local tumors at graft sites, measuring 0.5 and 1.0 cm in 2 of 4 recipients, 91 and 152 days after grafting. These recipients were 5 and 7 months old at death. One also had thymic lymphoma and generalized leukemia. A third recipient of this thymus developed generalized leukemia at 5 months of age, 90 days after grafting, with questionable thymic enlargement.

One of 4 recipients of a 50-day-old thymus developed generalized leukemia at 4½ months of age, 76 days after grafting, without tumors at graft sites.



From the thymus of AK/Z mice

FIG. 4. Same as Fig. 2. × 550.
FIG. 5. Same as Fig. 3. × 550.

Microscopic picture of the thymus of a virus-injected mouse at 58 days, judged to be not leukemic, is shown in Fig. 6. Bio-assay of thymus was negative for leukemic cells. In contrast, bio-assays of 68- and 73-day-old mice, clearly leukemic morphologically, were positive (Fig. 8,9). When replacement of normal thymus cells by large (leukemic) lymphocytes begins, it appears to proceed rapidly. An intermediate, conceivably "conditioned," phase is illustrated in Fig. 7. This shows the thymus of a virus-injected 65-day-old mouse with increased number of medium sized lymphocytes, which on bio-assay

TABLE II. Transplantation Assays for Neoplastic Cells in Thymuses of Virus-Injected AK/Z Mice.

Mouse assayed			Recipients					
Age (days) at Inj.	Age (days) at Assay	Thymus micro.	Age (days) at Graft	Age (days) at Death	Days after graft	Result of assay* Grafted L‡	Spont. L§	Neg.
2	35	N	58	148–210	90–152	3/4	1/4	—
2	36	N	62	178–212	116–150	—	4/4	—
2	36	N	62	187–206	125–144	—	4/4	—
2	50	N	57	133–194	76–135	1/4	3/4	—
2	50	N	57	157–171	103–114	—	4/4	—
1	52	N	36	104	68	—	—	2/2
1	52	N	36	104	68	—	—	2/2
1	53	N	32	137	105	—	1/1	—
2	56	N	36	104	68	—	—	2/2
3	57	N	36	92, 104	56, 68	1/2	—	1/2
3	58	N?	34	137	103	—	1/1	—
3	59	N	32	147	115	—	1/1	—
2	60	±?	43	92, 104	49, 61	1/2	—	1/2
2	60	N?	43	104	61	—	—	1/1
3	62	+	34	75	41	1/1	—	—
1	62	+	40	117	77	1/1	—	—
2	65	±?	39	98	59	—	—	2/2
2	65	+	39	98	59	—	—	1/1
1	66	atr	?	?	65	—	—	2/2
2	67	±?	?	?	65	—	—	2/2
2	67	—	45	95	50	1/1	—	—
3	68	+	40	60	20	1/1	—	—
3	73	+	45	67	22	1/1	—	—
2	74	+G†	?	?	23	2/2	—	—
2	74	+G	?	?	21, 34	2/2	—	—

* No. of mice/No. grafted.

† G = generalized leukemia.

‡ Generalized leukemia without thymic lymphoma, with or without tumor at graft site. All but 4 "positive" in this column had a tumor at graft site.

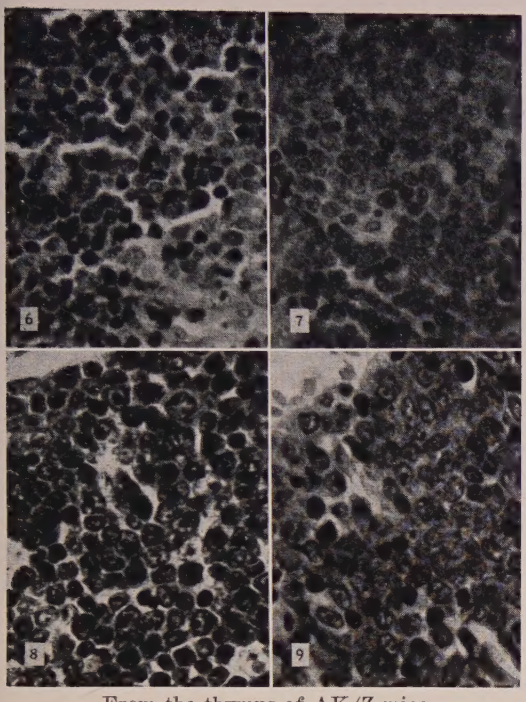
§ Thymic lymphoma with or without generalized leukemia, without tumor at graft site.

proved negative for autonomous leukemic cells. A closer study of this transition period with better cytologic technic is indicated.

All assays considered to be positive yielded local tumors at graft sites, with generalized spread but without thymic involvement, with the 2 following exceptions: One recipient of a 60-day-old thymus, and another of a 74-day-old thymus, developed generalized leukemia without thymic involvement and without local tumors at graft sites at 68 and 34 days, respectively, after grafting.

Worthy of note is development of "spontaneous" thymic lymphoma, in 18 of 22 mice receiving grafts of virus-injected but microscopically normal thymuses. These leukemic mice were 100 to 151 days old at death. Fig. 1 indicates that these leukemias were virus-induced, *i.e.*, the non-leukemic grafted thymuses carried virus. This was not unexpected, since it is known that virus persists in thymectomized mice and that non-leukemic AK mice of both sexes transmit the leukemia "factor" to their offspring(4,5,6).

Summary and conclusions. 1. Incidence of thymic leukemia in AK/Z mice injected as newborns with Gross leukemia virus (agent A) was 90% and the peak incidence of the disease occurred at 3-4 months of age. Incidence of leukemia in the controls was 73% with a peak occurrence at 8-12 months. 2. Thymuses from 52 sacrificed virus-injected AK/Z mice were examined at 13-72 days of age. No gross or microscopic abnormality was noted until about 50 days, when multiple foci of medium to large lymphocytes appeared in the cortex. These foci rapidly became confluent and at about 70 days of age, the thymus was transformed into a lymphomatous tumor. 3. Bioassays of 22, 57-74 day old thymuses from virus-injected mice disclosed autonomous leukemic cells in 10. Correlation of microscopic and bioassay diagnosis of malignant transforma-



From the thymus of AK/Z mice

FIG. 6. Virus-inj. 58-day-old mouse. Bio-assay negative. × 550.

FIG. 7. Virus-inj. 65-day-old mouse. Bio-assay negative. × 550.

FIG. 8. Virus-inj. 68-day-old mouse. Bio-assay positive. × 550.

FIG. 9. Virus-inj. 73-day-old mouse. Bio-assay positive. × 550.

tion was somewhat below 100%. Some thymuses, which were judged to be leukemic "in situ", were not transplantable. 4. Bioassays indicate the presence of virus in non-leukemic thymuses of virus-injected mice. 5. It is concluded that the pathogenesis of virus-induced thymic lymphoma is similar to that of "spontaneous" lymphoma in the high leukemic strain but occurs at an earlier age.

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Steroids and Carbohydrate Metabolism in the Domestic Bird.* (26369)

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Cortisone has frequently been used as a standard reference compound in the study of glucocorticoid activity in the mammal. In such studies, cortisone has invariably been ranked close to hydrocortisone in activity although slightly less potent(1-3). While numerous investigators have reported a high potency for hydrocortisone in the bird(4-7), few have attempted to compare the activity of hydrocortisone and cortisone. In some instances, cortisone has been utilized in studies in the bird(8,9), but no comparison with hydrocortisone was undertaken. Previous results from this laboratory have indicated that cortisone, in contrast with hydrocortisone, possesses little glucocorticoid activity in the bird(7). In addition, Stamler(4) reported a failure to induce hyperglycemia in the chick with cortisone at doses which were effective with hydrocortisone.

In view of an apparent lack of knowledge concerning the relative effectiveness of vari-

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† NSF Cooperative Fellow, 1959-1961.

‡ Adrenal corticoids were obtained through the courtesy of Dr. R. O. Stafford, Upjohn Co.

ous adrenal corticoids on carbohydrate metabolism of the bird it was felt that a comparison of certain steroids should be made with respect to their action upon blood glucose and liver glycogen in the chicken.

Materials and methods. Adult White Leghorn hens were used in the studies on blood glucose. The birds were kept in individual wire cages under conditions of constant temperature, humidity and light throughout period of experimentation. Food and water were supplied *ad libitum*. In each experiment birds were given daily intramuscular injections for periods of 14 days. The hormones were suspended in saline and Tween 80 and injected in a volume of 0.2 or 0.4 ml. Stilboestrol was given in oil solution. Blood glucose determinations were made according to the method of Nelson and Somogyi (10 and 11) on day one (before the initial injection) and on various days thereafter. Blood was obtained from the non-fasted bird by cardiac puncture.

Day-old Arbor Acres White Rock Vantress chicks were used in the glycogen study. The birds were placed in chicken brooders and kept there until placed on experiment. Chicks weighing between 300 and 600 g were starved for 24 hours prior to killing. All hormones were suspended in saline and Tween 80 and injected intramuscularly in a volume of 0.2 ml per injection. The total dose of hormone was divided into 4 equal injections spaced at 2 hour intervals. The birds were decapitated 2 hours following final injection and a sample of liver quickly removed and frozen in a dry ice-ether mixture. Frozen samples were weighed on a torsion balance and digested in hot 30% KOH. Glycogen concentration was determined by the anthrone procedure(12).

Results. Blood sugar and general changes. Hydrocortisone induced a marked and rapid hyperglycemia at the effective dosages of 2.5 to 10 mg per day (Fig. 1). The rise in blood sugar occurred in 24 hours and was accompanied by a marked polyuria, often within 2 to 4 hours after initial injection of the hydrocortisone. Marked polydypsia became apparent after 2 to 3 days of treatment. In general, the peak in the hyperglycemic response was reached in 1 to 3 days and was maintained throughout the treatment. All blood glucose levels returned to normal within 2 to 5 days after cessation of treatment.

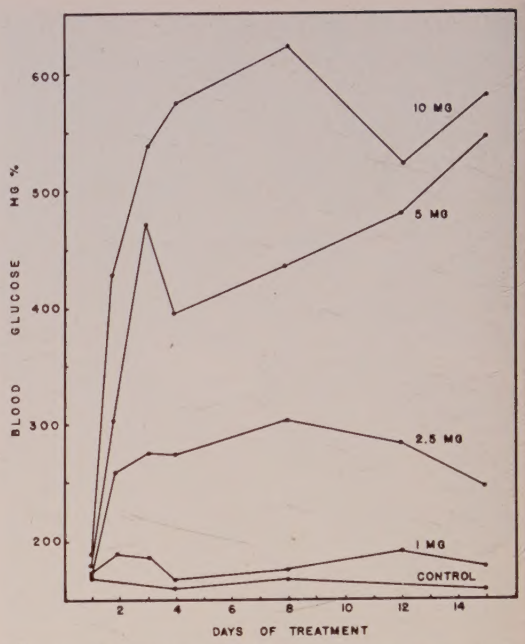

FIG. 1. Hyperglycemia in the hen following daily inj. of 1.0 to 10 mg hydrocortisone for 2 wk.

Only hydrocortisone and corticosterone produced a highly significant hyperglycemia of 300 mg % or more over the 14 day period of treatment (Table I). While 1 mg hydrocortisone induced a slight rise from 174 to 202 mg % blood glucose, 2.5 mg elevated blood glucose to a maximum value of 335 mg % and 10 mg elevated the sugar level to 651 mg %. Likewise, 5 mg corticosterone elevated the blood sugar level to 448 mg %.

In contrast to these results, neither cortisone nor desoxycorticosterone induced a comparable response when given at dosage levels as high as 40 to 50 mg per day. Cortisone showed a very slight hyperglycemic response in that blood sugar rose from 166 to 221 mg % and desoxycorticosterone caused a rise from 150 to 227 mg %. Stilboestrol failed to induce any hyperglycemic response at dose levels of 1 to 2 mg. Testosterone caused a decrease in blood sugar levels from 164 to 125 mg % at a daily dose of 20 mg, and from 190 to 161 mg % at a daily dose of 40 mg.

TABLE I. Blood Glucose Response to Various Hormones in the Hen.

Treatment	No. of birds	Blood glucose, mg/100 ml Initial	Maximum	Minimum
Control	10	170	175	154
Hydrocortisone				
1 mg	7	174	202	159
2.5 "	8	171	335	195
5 "	9	181	546	303
10 "	4	190	651	440
Corticosterone, 5 mg	5	159	448	241
Cortisone				
10 mg	3	190	203	177
20 "	6	174	199	161
40-50 "	15	166	221	161
Desoxycorticosterone acetate, 40 mg	5	150	227	179
Testosterone				
20 mg	6	164	169	125
40 "	4	190	166	161
Stilboestrol, 1-2 mg	5	181	193	168

However, when blood glucose values in the androgen-treated birds were corrected for increase in hematocrit, no significant differences were obtained in blood sugar levels following treatment with testosterone.

Stilboestrol and DCA produced no overall change in body weight. However, DCA caused a marked fluctuation in body weight throughout period of treatment. Changes of over 10% were seen within periods of 3 or 4 days. Testosterone produced a slight gain in weight throughout the experimental period. However, this was followed by a sharp weight depression upon termination of treatment. Cortisone and corticosterone at the doses used and hydrocortisone at doses above 2.5 mg caused body weight depression.

Oviposition was depressed by all hormones used except stilboestrol. Of the other hormones cortisone had the least depressing action.

Liver Glycogen. Hydrocortisone acetate, corticosterone and 11-dehydrocorticosterone induced significant deposition of glycogen in the fasting chick (Table II). Significant increases in the liver glycogen values were obtained with 25 μg of hydrocortisone acetate, 200 μg of corticosterone and 4 mg of 11-dehydrocorticosterone. Since the regression lines for these 3 compounds were somewhat comparable (Table II), it was possible to obtain an estimate of relative potency. If hydrocortisone is assigned an activity of 100, then corticosterone can be shown to possess an activity of 12.5 and 11-dehydrocorticosterone an activity of 1. On the other hand, neither cortisone acetate nor 11-desoxy-17-hydroxycorticosterone produced significant deposition of liver glycogen at doses as high as 16 mg. Thus, neither cortisone acetate nor 11-desoxy-17-hydroxycorticosterone were active in deposition of liver glycogen at dosages that were 640 times the effective dose of hydrocortisone.

Discussion. The present investigation has reaffirmed the observation that the bird is sensitive to glucocorticoid activity(4-7). Contrary to the findings of Brown, Brown and Meyer(8) who used cortisone, the dosage of hormone required to elicit a physiological change in the bird does not appear to be greater than that in the mammal if hydrocortisone is used as the glucocorticoid. Ol-

TABLE II. Liver Glycogen Response to Various Steroids in the Chick.

Treatment	No. of birds	Liver glycogen mg/100 g liver	Slope of curve
Control	59	248	
Hydrocortisone acetate			
25 μg	10	356 ± 29.6	4.1
50 "	10	662 ± 63.7	
100 "	9	738 ± 79.6	
200 "	11	888 ±124.3	
400 "	10	732 ± 41.6	
800 "	10	991 ±100.5	
Cortisone acetate			
4 mg	10	280 ± 35.5	.92
8 "	7	325 ± 56.4	
16 "	10	326 ± 41.4	
Corticosterone			
200 μg	10	348 ± 27.2	3.9
400 "	9	466 ± 50.6	
800 "	8	741 ± 67.0	
11-dehydrocorticosterone			
2 mg	10	296 ± 51.6	4.9
4 "	10	544 ±102.0	
11-desoxy-17-OH-corticosterone			
8 mg	10	246 ± 23.0	.88
16 "	10	290 ± 33.5	

son, Thayer and Kopp(1) using 440 μg of hydrocortisone found a liver glycogen deposition of 730 mg per 100 g of liver in the adrenalectomized rat. In the present study a total dose of 200 μg produced a glycogen deposition of 900 mg per hundred grams of liver. While the 2 experiments are not entirely comparable the results tend to indicate that the bird is no less responsive to glucocorticoids than the mammal.

The point that many investigators have failed to note is the fact that cortisone is virtually inactive as a glucocorticoid in the bird. Thus, the observation of Brown *et al.* must be restricted to cortisone. Certainly enormous doses of this hormone are required to elicit a physiological change in the bird. However, a failure to respond is not obtained for all glucocorticoids. Indeed, hydrocortisone and corticosterone are highly active in the bird. Therefore, it is important that studies involving the use of glucocorticoids in the bird employ corticoids which are physiologically active in the bird. This failure of the chick to respond in a manner comparable to that seen in the mammal has also been noted for the progestogens(13).

The depressant activity of testosterone upon blood glucose levels in the hen has previously been demonstrated(14). It appears that this effect is not a result of direct interference in carbohydrate metabolism of the bird but is, rather, an indirect effect resulting from an increase in hematocrit induced by treatment with testosterone.

Summary. The effects of a series of steroids on the carbohydrate metabolism of the bird were studied using blood glucose and liver glycogen as endpoints. The highly active compounds in the hyperglycemic reaction were hydrocortisone and corticosterone and the active compounds in the liver glycogen test were the above two plus 11-dehydrocorticosterone. Cortisone was inactive in both tests and 11-desoxycorticosterone acetate was tested only in the hyperglycemia reaction and was inactive. If hydrocortisone is assigned an activity of 100 in the glycogen test, then corticosterone shows an activity of 12.5 and 11-dehydrocorticosterone an activity of 1. In direct contrast to the mammal, cortisone is inactive in the bird. Testosterone appears to lower blood glucose but this apparently is a result of an increase in red blood cell number rather than an absolute drop in glucose present in the blood. Stilboestrol had no effect on blood glucose levels.

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Interaction of Myxoviruses with Human Blood Platelets *in vitro*. (26370)

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(Introduced by A. L. Olitzki)
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The interaction of myxoviruses with red blood cells has been the subject of extensive investigation. The kinetics of adsorption, its requirements of cations and the kinetics of elution are known(1). The distribution and chemical identity of the virus receptors

have also been established(2). The inability of the red blood cells to adsorb additional virus(3) is associated with a change in electrophoretic mobility of the red blood cell(4), and has been ascribed to loss of receptors due to the action of a viral enzyme(5). This enzyme is similar to the receptor destroying enzyme (RDE) produced in bacterial cultures(6). Myxoviruses have been shown to be adsorbed onto white blood cells, even in absence of cations(7). When leukocytes are treated with RDE, virus particles are still adsorbed(7), although to a lesser degree. Studies of the elution of myxoviruses from white blood cells have given inconsistent results(7,8,9). In recent work it has been shown that blood platelets also adsorb some myxoviruses and become agglutinated(10, 11). In the present study the adsorption and elution of myxoviruses to and from blood platelets is compared to that occurring with red blood cells.

Materials and methods. Platelets. 18 ml of human venous blood were drawn into 2 ml of a solution of 1% Na_2 sequestrene, 1% triton and 0.7% NaCl. Siliconized (Dow Corning D.C. 200) glassware was used throughout. After centrifugation at 1000 rpm for 15 minutes, the plasma was separated and centrifuged at 4°C for 15 minutes at 2000 rpm. The suspension was then diluted 40 fold with 0.15 M phosphate buffered saline (pH 7.2) and washed twice by centrifugation at 2000 rpm for 15 minutes at 4°C. The final sediment was suspended in buffered saline to a concentration of about 5×10^5 platelets per cmm. *Red blood cells.* The red blood cells obtained after the first centrifugation of the blood sample were washed twice in buffered saline by centrifugation at 4°C at 2000 rpm for 7 minutes. A final concentration in buffered saline of 83.000 cells per cmm was prepared. At these respective concentrations platelets and red cells have approximately equal total cell surfaces; this is required for comparison of kinetics of adsorption and elution between the 2 types of cells. The surface of a red blood cell according to Ponder (12) is 163 μ^2, and the surface of a platelet was calculated to be 28.3 μ^2 (assuming a spheric form and a radius of 1½ μ(13). *Virus.* Influenza A, strain FM_1, and Newcastle Disease Virus (NDV), strain HP(14) were used. Stock virus was prepared by injecting 0.2 ml of 10^{-3} dilutions of infected allantoic fluid into the allantoic sac of 9 days old chick embryos. The infected allantoic fluid was harvested after 48 hours of incubation at 36°C and stored at –20°C. Before use the virus was purified and concentrated by one cycle of adsorption and elution according to Francis and Salk(15). Dialyzed virus was prepared according to Ginsberg and Blackmon(7). The potency of virus suspensions was determined by hemagglutination: 0.2 ml of a 1% suspension of chick red blood cells was added to a series of dilutions of the virus (1:1.5) in 0.2 ml in 0.15 M phosphate buffered saline (pH 7.2) and after suitable incubation the hemagglutination titer was read according to Salk(16). *Receptor destroying enzyme.** Titration of the enzyme was performed in a solution containing 1 g $CaCl_2$, 9 g NaCl, 1.203 g H_3BO_4, 0.052 g $Na_2B_2O_7 \cdot 10H_2O$ in 1000 ml of distilled water. To 0.25 ml of dilution of the enzyme were added 0.25 ml of a 1% suspension of red blood cells. After incubation for 30 minutes at 37°C, 0.2 ml containing 10 hemagglutinating doses of influenza virus per ml was added. After incubation at 4°C for one hour the titer of enzyme was determined as the highest dilution which inhibited the agglutination of red blood cells by virus. For receptor destruction, 10 units of RDE were added to a 1% suspension of red blood cells or to a corresponding quantity of platelets. After incubation for 30 minutes at 37°C the cells were washed by centrifugation at 2000 rpm for 10 minutes. *Electrophoretic mobility.* Electrophoretic measurements(17) were carried out at 25°C in a Northrop-Kunitz type micro-electrophoresis apparatus, equipped with reversible electrodes (copper in saturated copper sulfate) and mounted on the stage of a phase-contrast microscope. All measurements were taken in the lower stationary layer. Measurements for each mo-

* Behringwerke, Marburg-Lahn.

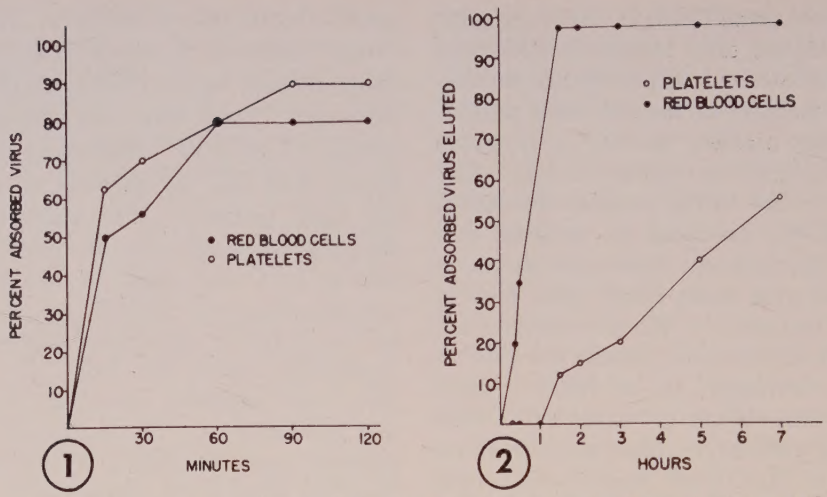

FIG. 1. Adsorption of influenza FM_1 virus to platelets and red blood cells at 4°C.
FIG. 2. Elution of influenza FM_1 virus from platelets and red blood cells at 37°C.

bility determination were performed on 20 platelets, 10 before and 10 after reversal of the direction of the field. *Adsorption and elution of viruses.* To 0.25 aliquots of cell suspension, 0.25 ml of virus suspension was added (hemagglutination titer influenza FM_1-1:1250 and NDV HP-1:700). The tubes were incubated at a desired temperature, and after consecutive time periods centrifuged at 2000 rpm for 10 minutes. The supernatants were titrated for the hemagglutinative titer, and the percentage of virus adsorbed calculated. Following an adsorption period of 90 minutes at 4°C, the cells were spun down, washed, resuspended in saline and then incubated in a 37°C water bath for varying periods of time. After centrifugation, the hemagglutination titer of the supernatants was determined and the percentage of virus eluted calculated.

Results. Adsorption. The time-curve of adsorption of NDV and influenza viruses to blood platelets at 3 temperatures, 4°, 23° and 37°C, resembled that observed for red blood cells. Fig. 1 represents comparative curves of adsorption of influenza virus to red blood cells and to platelets at 4°C. Similar curves were obtained when the reaction proceded at 23° and at 37°C. Adsorption of influenza virus to platelets was maximal at 4°C, while that of NDV was maximal at 37°C, similar to adsorption onto red blood cells.

Cations. The influence of cations on the adsorption was studied in an isotonic glucose-NaCl solution containing varying NaCl concentrations, using dialyzed virus brought to the same ionic strength. Table I shows that adsorption of influenza virus to red blood cells ceases at a concentration of 0.01 M NaCl, while the virus was retained by platelets even when concentration of NaCl was as low as 0.0025 M.

Elution. There was a striking difference in the kinetics of elution of the 2 myxoviruses from the red blood cells on the one hand and from the platelets on the other. Under comparable conditions less virus was eluted from platelets than from red blood cells and the process of elution from platelets took a much longer time. This difference for influenza virus is seen clearly in Fig. 2.

Red blood cells from which influenza virus had been eluted were unable to adsorb additional virus, whereas platelets still adsorbed small quantities of the same virus after a single cycle of adsorption and elution (Table II).

Similar results were obtained when the receptors on red blood cells and on platelets were destroyed by RDE. Following the treatment with this enzyme the red blood cells became incapable of binding influenza

TABLE I. Adsorption of Influenza Virus to Red Blood Cells and to Platelets at Decreasing NaCl Concentration.

	Molar conc. of NaCl				
	.155	.100	.050	.010	.0025
	% of virus adsorbed*				
Platelets, 5×10^5/mm^3	87	87	87	56	56
Red blood cells, 8.3×10^4/mm^3	70	70	56	0	0

* % of hemagglutination units of virus which disappeared from the supernatant after adsorption for 90 min. at 4°C.

virus, while the platelets still adsorbed a small amount (Table III).

Treatment of platelets and of red blood cells with RDE, reduced the electrophoretic mobility of both, almost to the same extent (Table IV).

Discussion. The results of the experiments described here indicate that the receptors for myxoviruses on red blood cells and on platelets may be identical. This contention is supported by the similarity of extent and kinetics of adsorption of NDV and influenza virus to both types of blood cells, as well as by the reduction of electrophoretic mobilities of these cells by RDE treatment. In view of receptor identity, the incomplete elution of virus from the platelets as compared to the almost complete elution from the red blood cells may be explained by assuming that virus is incorporated by the platelets. Danon *et al.*(18) demonstrated in electronmicrographs influenza virus particles inside vacuoles of the platelets.

The ability of the platelets to remove virus from the medium in absence of cations, and even after previous adsorption of virus and its elution, or after RDE treatment demonstrates further differences in the interaction

TABLE II. Adsorption of Influenza Virus to Red Blood Cells and Platelets from Which Influenza Virus Had Been Eluted.

	% virus adsorbed	
	Previously unadsorbed	Following elution
Platelets, 5×10^5/mm^3	80	30
Red blood cells, 8.3×10^4/mm^3	70	0

of viruses with platelets and red blood cells. This, too, may be explained by uptake of virus particles by the platelets. A similar consideration may be valid for the white blood cell which incorporates virus particles in absence of cations(7) and following RDE treatment (7). Finally, the delay in initiation of elution of influenza and NDV viruses from platelets and its slower rate may be related to morphological and biochemical changes occurring in the platelets at 37°C. At this temperature viscous metamorphosis occurs(19), resulting in loss of the hyalomer and subsequent aggregation of platelets(20). Thus, virus particles trapped in vacuoles situated in the subsurface of the platelets could be gradually released following disintegration of the hyalomer. Later, when compact platelet aggregates are formed, some virus parti-

TABLE III. Adsorption of Influenza Virus to Red Blood Cells and to Platelets Following Treatment with RDE.

	% virus adsorbed	
	Untreated	After RDE treatment
Platelets, 5×10^5/mm^3	70	20
Red blood cells, 8.3×10^4/mm^3	60	0

TABLE IV. Effect of RDE Treatment on Electrophoretic Mobility (μ/Sec./V/cm) of Platelets and Red Blood Cells.

	Untreated	RDE treated
Platelets	−1.20	−0.53
Red blood cells	−1.00	−0.55

cles trapped in vacuoles inside these aggregates, are not liberated into the supernatant even when the incubation is prolonged. This would explain the incomplete elution of virus from the platelets even after 7 hours of incubation at 37°C.

Summary. 1. Influenza and Newcastle Disease Viruses are adsorbed to red blood cells and to platelets in a similar manner. Evidence is presented indicating that there exists similar virus receptors on the surface of the red blood cells and of the platelets. 2. Virus is adsorbed to platelets in the absence of cations as well as after its previous

elution and after RDE treatment of platelets. Furthermore elution of the virus from platelets is much slower and less complete than that from red blood cells. These phenomena may be due to incorporation of virus particles into the platelets.

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Relationship of Human and Bovine Strains of Myxovirus Para-Influenza 3. (26371)

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The first recognized strain of para-influenza 3 virus was the hemadsorption type 1 (HA-1) virus recovered from children with pneumonia and febrile upper respiratory illness(1). A virus (SF-4) with similar properties of para-influenza 3 virus was recovered from cattle with respiratory disease (shipping fever)(2). The original serological comparison of these human and bovine viruses indicated they were antigenically indistinguishable(3). Subsequent studies by Hamparian and Hilleman, however, showed the viruses could be differentiated by use of serum from intranasally infected guinea pigs (4). The work reported here confirms and extends their findings.

Materials and methods. Virus. The human, HA-1, and bovine, SF-4, strains of the virus used to infect the guinea pigs were grown, respectively, in monkey and bovine kidney tissue cultures. The isolation history and passage of these strains has been reported (1,2). The HA-1 pool had an infectivity of $10^{6.5}$ per ml and the SF-4 pool of 10^{7} per ml. In addition, bovine isolates from different parts of the United States, as well as human isolates from 1955-1959 in Washington, D.C., and from Canada, France, England and Australia, were studied.

Soluble antigens. Preparation of HA-1 and SF-4 soluble antigens is described by Cook, *et al.*(5).

Inoculation of guinea pigs. All guinea pigs were prebled and no hemagglutination inhibition (HI) or complement-fixing (CF) antibodies could be demonstrated with either human or bovine strain antigens. Sera of several animals were also tested for presence of

TABLE I. Antibody Response of Guinea Pigs Infected with Human and Bovine Para-Influenza 3 Viruses.

Days following infection	Infection with HA-1 (human strain)				Infection with SF-4 (bovine strain)			
	HI		Neut.		HI		Neut.	
	HA-1	SF-4	HA-1	SF-4	HA-1	SF-4	HA-1	SF-4
0	<10†	<10	<10	<10	<10	<10	<10	<10
14*	80	10			10	20		
22	640	40	160	40	20	160	<10	20
0	<10	<10	<10	<10	<10	<10	<10	<10
14*	20	<10			<10	10		
22	320	20	160	10	10	80	<10	40
0	<10	<10			10	<10		
14*	40	20			<10	10		
22	320	80			10	160		
0	<10	<10			<10	<10		
14*	<10	<10			10	40		
22	80	<10			160	640		

* Soluble antigen (0.5 ml) inoculated intraper.
† Reciprocal of antibody titers with indicated strain.

neutralizing antibodies and were negative.

The 2 groups of guinea pigs used were housed in separate buildings and were inoculated and tended by separate personnel. They were given 0.2 ml of the respective viruses intranasally. Fourteen days later the guinea pigs were bled and given a 0.5 ml dose, intraperitoneally, of the respective undiluted soluble antigen. Eight days following inoculation of the soluble antigen all animals were bled out.

Human sera. The human sera were from children who had experienced primary infections and some reinfections with para-influenza 3 virus during nursery outbreaks of respiratory disease(6).

Bovine sera were from calves which experienced inapparent infections in nature(7). One calf (#44) was subsequently challenged with the SF-4 strain of the virus.

Serological procedures. Hemagglutination inhibition (HI). All sera were prepared and tested as described by Abinanti, *et al.*(8). Essentially this consisted of adsorption of sera with kaolin followed by adsorption with bovine erythrocytes. Bovine erythrocytes were used as they produced tests which could be easily read. *Neutralization (N).* The neutralization test used has been described by Chanock, *et al.*(1). The test was performed in the usual way and readings were made by means of the "hemadsorption" technic(9).

Results. Guinea pig sera. Table I shows representative responses of 4 guinea pigs in each group to infection. HI antibody to the homologous strain of virus frequently appeared earlier and was 4-fold or greater than antibody for the heterologous strain. Levels of neutralizing antibody were also higher for the homologous than the heterologous strain. There was an increase in antibody titer from the 14th to the 22nd-23rd day.

Human single and multiple infection sera. Results of neutralization tests with the HA-1 and SF-4 strains of virus are shown in Table II. Responses of 4 of the 5 children undergoing first infection were similar to those of the guinea pigs, *i.e.,* moderate to high levels of antibody were observed for the homologous HA-1 strain, while low levels or no antibody for the SF-4 strain was detected. One child (J. Fl.) developed the same level of antibody to both antigens.

Following second infection with para-influenza 3 virus, high levels of HA-1 antibody were produced and one child (D. Pit.) developed a low level of antibody to the bovine strain. The sera of 2 children (S. Tate and D. Pit.) were also tested by HI and no bovine strain antibody was observed following the first infection; however, it was present following the second infection of both children at essentially the same level as HA-1 antibody.

It is of interest that the presence or ab-

TABLE II. Para-Influenza 3 Neutralizing Antibodies in Children during 1st and 2nd Natural Infections.

Individual	Test virus, para-influenza 3	Reciprocal of neut. antibody titer: 1st infection Pre	1st infection Post	2nd infection Pre	2nd infection Post
S. Tate*	Human‡	<8	64	64	256
	Bovine§	<8	<8	<8	<8
D. Pit†	Human	<8	32	64	1024
	Bovine	<8	<8	<8	16
M. St.	Human	<8	512	—	—
	Bovine	<8	<8	—	—
J. Br.	Human	<8	1024	—	—
	Bovine	<8	64	—	—
J. Fl.	Human	<8	1024	—	—
	Bovine	<8	1024	—	—

* 6 mo interval between infections.
† 3 " " " "
‡ 100 to 320 TCD_{50} in test.
§ 32 TCD_{50} in test.

sence of bovine strain antibody did not appear to be related to the level of HA-1 antibody. (Table II (M. St., J. Br., and J. Fl.).

Bovine infection sera. It was not possible to secure sera from cattle which had been under as close medical scrutiny as the children just described. However, we did have several pre- and post-infection sera from dairy calves that were infected in nature. One of them was subsequently challenged with an aerosol of SF-4 strain of the virus. These sera were tested only for the presence of HI antibodies. While antibody response to both antigens was poor, homologous levels were higher. The one calf which was challenged with the SF-4 strain of the virus produced high levels of antibody to both antigens.

Identity of human and bovine isolates. It was of interest to determine whether other human and bovine isolates had the same antigenic properties as the prototype strains. The human strains of para-influenza 3 virus were recovered from individuals in Washington, D.C. (from 1955 through 1959), Cincinnati(10), California(11), Canada(12), France(13), England(14), and Australia (15). The bovine strains of the virus were isolated from cattle in Maryland, Pennsylvania(8), Kansas(16), California(17), and New York(18). Each of the strains of virus was used as antigen (4-8 units) in the HI test against dilutions of pooled HA-1 and SF-4 strain guinea pig serums, aliquots of the same sera reported earlier in this paper. Each of the human and bovine isolates resembled its respective prototype (Table III). The human isolates were inhibited by HA-1 serum at a 4-fold or higher titer than with SF-4 serum. A similar specificity was observed for the bovine strains with the SF-4 serum. While there is evidence that the human and bovine strains are related, it would appear that 2 antigenically distinct species specific groups exist.

Discussion. Abinanti and Huebner(3) observed no differences in HI, CF or N antibody responses to the HA-1 or SF-4 strain antigens with human, bovine, rabbit or rooster serums. The work of Hamparian and Hilleman(4), has explained these differences. They observed that guinea pigs developed a specific response following infection but that this specificity was lost following repeated exposure to the same virus. A review of the earlier report(3) suggests that the human and bovine serums came from individuals that had prior experience with the virus (as evidenced by HI titers of 1 in 10 or greater in the acute or preinoculation serums), while the rabbits and roosters were given multiple doses of virus.

From these data it is not possible to determine whether this rise in antibody from the 14th to 22nd-23rd day was due to inocu-

TABLE III. Hemagglutination Inhibition Antibody Titers of Strain Specific Guinea Pig Serum Pools when Tested with Human and Bovine Strains of Para-Influenza 3 Virus from Various Parts of U. S., Canada, and Foreign Countries.

	No. of strains showing indicated pattern	Reciprocal of HI titer: HA-1 guinea pig serum pool	SF-4 guinea pig serum pool
Human	5	80	20
	6	160	20
	7	160	40
	5	320	80
Bovine	1	40	320
	4	20	160
	1	<20	80
	1	<20	40

lation of soluble antigen or reflects the temporal course of antibody development. The first possibility seems unlikely, since the ether-treated soluble antigen preparation which was absorbed twice with erythrocytes was free of demonstrable hemagglutinin. Such a soluble antigen would not be expected to stimulate antibody for the viral hemagglutinin.

Pools of sera from each of these groups of guinea pigs were also tested for presence of complement-fixing antibody with HA-1 and SF-4 tissue culture grown viruses. In each instance serum from HA-1 or SF-4 guinea pigs fixed complement with both antigens, but there was at least a 4-fold greater antibody response to the homologous virus.

An interesting observation was the diverse neutralizing antibody response shown by the 3 children (M. St., J. Br., and J. Fl.) following their first infection with para-influenza 3 virus. The factors which influenced the heterologous response in these circumstances are not known. It is possible that prior experience with other myxoviruses may be a factor.

Based on the available evidence it would appear that para-influenza 3 strains do not cross species boundaries. Additional evidence that human and bovine strains differ was provided when the human virus failed to infect a calf when given by aerosol(3). Subsequent challenge of the calf with a bovine strain resulted in infection. It is clear, however, that additional isolates from both man and cattle must be examined before concluding that infection may not occasionally cross species boundaries.

Summary. Guinea pigs infected intranasally with human (HA-1) and bovine (SF-4) strains of para-influenza 3 virus produced 4-fold or greater HI, N and CF antibodies to the homologous strain than to the heterologous strain. Children and cattle showed similar species-linked responses following infection with para-influenza 3 viruses. Human and bovine strains of the virus originating from various states and foreign countries and isolated in different years were shown to react in the same manner as the HA-1 and SF-4 prototype strains.

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Action of Acetazolamide-Reserpine Co-treatment on Metacorticoid Hypertension.* (26372)

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The clinical effectiveness of chlorothiazide and its congeners in treatment of hypertension has been demonstrated by a majority of the investigators in this field. The history, modes of action and efficacy of these drugs as antihypertensives have been reviewed extensively at two recent Hahnemann symposia(1,2). In contrast, the role of other types of diuretic drugs in treatment of hypertension has not been thoroughly explored. Although some reports indicate that acetazolamide does not share antihypertensive activity(3,4), other studies, such as the finding that acetazolamide augments antihypertensive action of mecamylamine(5), suggest that this potent carbonic anhydrase inhibitor may deserve a place in hypertension therapy.

The purpose of the present investigation was to study the effect of acetazolamide alone and in combination with reserpine on blood pressure of metacorticoid hypertensive rats. Chlorothiazide alone and in combination with reserpine was included for comparative purposes. The action of acetazolamide-reserpine co-treatment on development of left ventricular hyperthrophy was also assessed in this disease model.

Methods and materials. Metacorticoid hypertension was produced in male albino Sherman rats by a microcrystalline aqueous suspension of desoxycorticosterone acetate (DCA), 10 mg/0.2 ml, injected subcutaneously 5 days a week for 3 weeks. During this time, the animals were given 0.85% sodium chloride drinking fluid *ad libitum* and were maintained on a basal diet of Purina Laboratory Chow. In the metacorticoid period (the interval of time following corticoid treatment), the rats drank tap water. Body weights of the animals at start of the DCA-saline regimen ranged from 60-89 g. Indirect systolic blood pressure readings were taken at weekly intervals using the foot densitometer method(6). All indirect readings were taken approximately 2 hours prior to dosing. The compounds employed, routes of administration and dosage schedules are shown in Fig. 1 and 2. Gavage volumes were kept constant at 2.5 ml/kg body weight. Gavage treatments were administered once/day, 5 days/week. In comparing the effects of gavage and drug diet treatments, it should be noted that the rats on a 0.001% drug diet ingested approximately 1 mg drug/kg body weight/day. Eighty-four rats, 12/group, were employed in the first experiment (Fig. 1) and 4/group were autopsied at weekly intervals after 1, 2 and 3 weeks of treatment. In the second experiment (Fig. 2), 30 rats were employed, 6/group, and all animals were terminated after 3 weeks of treatment. Cardiac hypertrophy in metacorticoid hypertensive rats was characterized by determining ventricular ratios; *i.e.*, weight of left ventricle and whole septum/weight of the remaining free part of right ventricle.

Results. The average antihypertensive effects of acetazolamide, reserpine, and different combinations of the 2 drugs are shown in Fig. 1. Evaluation of these data by analysis of variance revealed that the effect of acetazolamide on systolic blood pressure of hypertensive rats was significant ($P<.01$). The regression of blood pressure on the dosage of reserpine employed (0-0.001% in diet) was likewise significant at the 1% probability level. The regression coefficient equalled −2.18 mm Hg/mg reserpine/kg of food. Combined administration of acetazolamide and reserpine resulted in algebraic summation of

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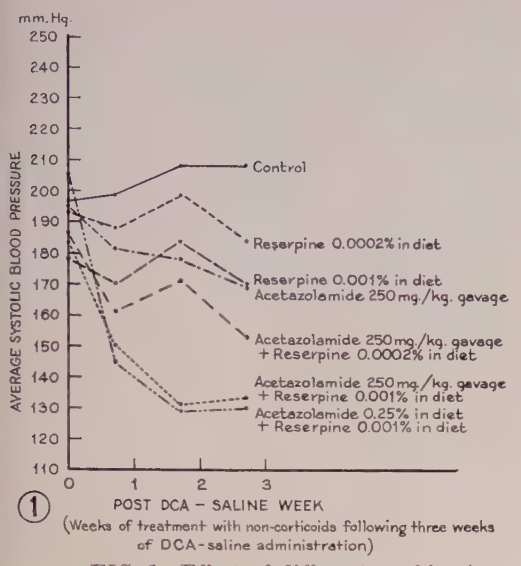

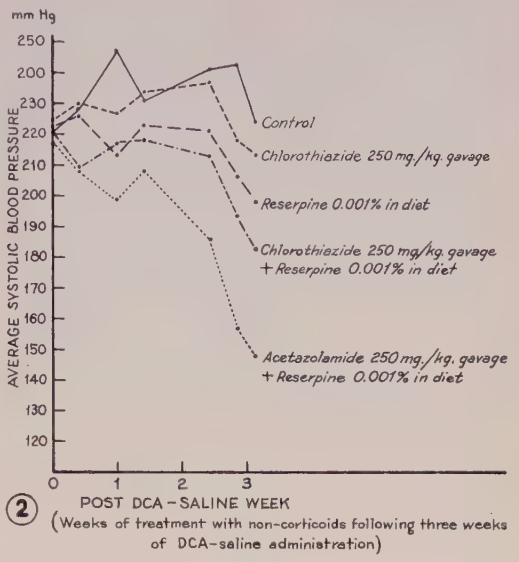

FIG. 1. Effect of different combinations of acetazolamide and reserpine on blood pressure of hypertensive metacorticoid rats. At 5% probability level, all blood pressures of treated animals were significantly less than control.

FIG. 2. Effect of combinations of chlorothiazide, acetazolamide and reserpine on blood pressure of hypertensive rats. At 5% probability level, all blood pressures of treated animals except those receiving chlorothiazide alone were significantly less than control.

the effects of the 2 drugs on systolic blood pressure. The difference between acetazolamide-reserpine co-treatments and either reserpine or acetazolamide alone was significant ($P<.05$). The interaction between number of weeks and treatments was not significant with any of the drug regimens.

Fig. 2 compares the effect of acetazolamide-reserpine and chlorothiazide-reserpine co-treatments on blood pressure of metacorticoid hypertensive rats. The effect of chlorothiazide alone on systolic blood pressure was not significant ($P>.05$) whereas reserpine alone showed a significant action. The greater reduction in blood pressure of the hypertensive rats which received a combination of chlorothiazide and reserpine as compared to the animals which received only reserpine is attributed to an additive effect since the interaction between the 2 drugs was not significant. Acetazolamide-reserpine co-treatment was associated with a significant reduction in blood pressure from control and from the means of all other treatments ($P<.05$).

The typical unilateral left cardiac hypertrophy associated with metacorticoid hypertension can be characterized by means of a ventricular ratio; *i.e.*, weight of left ventricle and whole septum/weight of right ventricle. In examining the data of the present study by analysis of variance, no statistical evidence was found for concluding that ventricular ratios changed in relation to number of weeks on drug treatment. Average ventricular ratios of treated and untreated hypertensive rats are shown in Table I. For comparative purposes, mean ventricular ratios of 18 normotensive control rats are also pre-

TABLE I. Ventricular Ratios (Weight of Left Ventricle and Whole Septum/Weight of Right Ventricle) of Untreated and Treated Hypertensive Rats and Untreated Normotensive Rats.

Treatment	Mean	95% confidence limits
Hypertensive control	4.23	4.00-4.46
Acetazolamide (A), 250 mg/kg	3.86	3.63-4.09
Reserpine (R), .001% diet	3.77*	3.54-4.00
A, 250 mg/kg + R, .001% diet	3.67*	3.44-3.90
Reserpine, .0002% diet	3.85	3.62-4.08
A, 250 mg/kg + R, .0002% diet	3.78*	3.55-4.01
A, .25% diet + R, .001% diet	3.78*	3.55-4.01
Normotensive controls	3.38*	3.19-3.57

* Significantly less than hypertensive control at 5% probability level.

sented. As may be noted, ventricular ratios of the metacorticoid rats receiving acetazolamide-reserpine co-treatment were significantly less than ratios of the control hypertensive rats. Analysis of the data by means of Dunnett's multiple comparison procedure (7) revealed that all of the mean ventricular ratios of the treated rats less than 3.84 are significantly smaller than the mean of 4.23 for the untreated hypertensive control rats.

Discussion. The absence of a significant reduction in arterial blood pressure following repetitive administrations of chlorothiazide to hypertensive rats attests to the difficulty encountered experimentally in demonstrating an antihypertensive action for this type of compound. The summary table of Sturtevant(8) indicating that chlorothiazide lowers the blood pressure of metacorticoid rats cannot be assessed since supporting data were not presented. The augmented antihypertensive action of reserpine-chlorothiazide co-treatment over that produced by either drug alone is in accord with the usual clinical finding(1,2). The pronounced antihypertensive action of acetazolamide-reserpine combination treatment was striking. The sustained hypotension encountered following repetitive co-treatments was of a greater magnitude than that previously recorded under identical experimental conditions after repetitive administrations of ganglioplegic and vasodilator agents. The interesting finding that acetazolamide-reserpine co-treatment tended to obviate development of left ventricular cardiac hypertrophy indicates that the combination therapy exerted a desirable effect on both the hemodynamic and the morphologic disturbances associated with metacorticoid hypertension.

Summary. An increased antihypertensive action of a combination of reserpine and acetazolamide over that produced singly by either drug was demonstrated in metacorticoid hypertensive rats. Acetazolamide alone had a moderate antihypertensive action, and the enhanced reduction in blood pressure following co-treatment was an additive rather than a potentiated effect. The typical hypertensive left ventricular cardiac hypertrophy was significantly reduced in the metacorticoid rats receiving acetazolamide-reserpine.

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Tolerance of Skin Homografts Induced in Adult Mice by Multiple Injections of Homologous Spleen Cells.* (26373)

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It seems now well established that acquired immunological tolerance of tissue homografts can be induced in adult mice of certain strains closely related genetically. For example, tolerance of male skin isografts was obtained in adult female mice of C57Bl (Subline 1) and A strains by a single intravenous injection of

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approximately 20 million spleen cells taken from male donors, or by placing the females in celomic parabiosis with isologous males(1). Similarly, adult Z (C3H) strain mice less than 1 month of age were made tolerant of (Z × Ce)F_1 hybrid skin homografts by intravenous injection of viable spleen cells taken from donors of the (Z × Ce) F_1 hybrid cross(2). Finally, mice of the Z (C3H) strain were made tolerant of either (A × Z) F_1 hybrids or Ce strain mice when animals of these strains were joined in celomic parabiosis (3). In the case of parabiosis it was also demonstrated that the time during which parabiosis had to be maintained to induce lasting tolerance varied with the strains of mice in parabiosis and apparently with degree of histocompatibility difference between the 2 strains(4). Thus, while only 4 days of parabiotic union between female and male mice of either A or C57Bl (Subline 1) strain were sufficient to abrogate immunological rejection reaction in the females to skin grafts taken from the male partner, 30 to 45 days days of parabiosis between Z(C3H) and homologous (A × Z) F_1 hybrid mice were necessary to permit establishment of tolerance of (A × Z) F_1 hybrid skin homograft in the Z (C3H) strain parabionts. Similar findings were obtained in case of parabiosis between Z (C3H) and Ce animals, although in this instance while only 27 days of parabiosis were necessary to produce tolerance of Ce skin homografts in the Z (C3H) partners, 64 days were necessary to induce tolerance of Ce skin in the Z (C3H) strain parabiont.

We interpreted these results to indicate that tolerance thus produced resulted from a continuous exchange of transplantation antigens in form of intact cells between both members of the pair in such amounts as to inhibit the homograft reaction. This state of nonreactivity could then permit establishment of cells capable of replication in the respective hosts, and these by producing antigen would be sufficient to maintain the tolerant state. On the basis of these observations it was postulated that were it technically possible to inject large enough numbers of viable cells intravenously with sufficient frequency over a sufficiently long period it might be possible to produce lasting tolerance by injection of homologous adult cells in adult recipients.

In the experiments reported here attempts have been made to simulate the parabiosis phenomenon and induce tolerance in mice subjected to repeated injections of large numbers of homologous viable spleen cells. The results can be interpreted as substantial support for our hypothesis.

Method. Mice of the Z (C3H) strain and (A × Z) F_1 hybrids of both sexes were used. Two-month-old mice of the Z (C3H) strain were repeatedly injected with viable spleen cells from homologous donors of the (A × Z) F_1 hybrid cross either by the intraperitoneal route alone, or alternatively by the intravenous route and the intraperitoneal route. The method of preparation of the spleen cells was as previously described(5). Mice to be injected were divided into groups according to number of spleen cells injected, duration of treatment and route of administration (Table I). Group I received a total of approximately 200 million viable spleen cells taken from donors of the (A × Z) F_1 hybrid cross administered in 4 intraperitoneal injections during 1 week. Group II received a total of approximately 1 billion 300 million cells of the same hybrid strain in 3 weekly intraperitoneal injections during 4 to 6 weeks. Group III received a total of approximately 500 million spleen cells in 3 weekly intraperitoneal injections over a period of 7 to 11 weeks. Group IV received a total of 1½ billion spleen cells injected over a period of 7 weeks. In this instance injections were performed daily during the first 2 weeks alternating the intravenous and intraperitoneal route and twice weekly during 5 consecutive weeks. Group V was not treated and remained as controls.

One day after the last injection experimental as well as control animals received a full-thickness abdominal skin homograft taken from (A × Z) F_1 hybrid donors. All donor and recipient mice were of the same sex. The technic of skin transplantation was the same as previously described(5) consist-

TABLE I. Induction of Immunological Tolerance in Z (C3H) Strain Mice Repeatedly Injected with Viable Spleen Cells Taken from (A × Z) F_1 Donors.

Group	Total No. of spleen cells inj. (millions)	Duration of treatment (wk)	No. of Z (C3H) mice accepting (A × Z) F_1 hybrid skin homograft/No. of mice grafted
I	200*	1	0/22
II	1,300†	4– 6	2/22
III	500†	7–11	1/31
IV	1,500‡	7	6/7
V	none	—	0/42

* 4 injections intraper.
† 3 intraper. inj./wk.
‡ Intraper. and intrav. (for schedule of treatment, see text).

ing essentially in transfer of a full-thickness abdominal piece of skin measuring 2 × 2 cm from the donor to the back of the recipient. The graft was turned 180° to facilitate determination of subsequent success or failure of the graft. In such grafts hair on successful grafts grows in a direction opposite to that on the skin of the host. Grafts were inspected 3 times a week for not less than 4 months before a final determination of success or failure of the graft.

After operation grafted mice were housed individually in plastic cages and fed Purina Laboratory Chow and tap water.

Results. The results are recorded in Table I. Mice of Group I rejected the homologous skin grafts in all instances. However, in mice of Group II pretreated with 1 billion 300 million homologous spleen cells intraperitoneally over a period of 4 to 6 weeks 2 of 22 (9%) accepted the skin grafts taken from (A × Z) F_1 hybrid donors. Only 1 of 31 mice of Group III receiving intraperitoneal injections of approximately 500 million homologous spleen cells over a longer period of time (7 to 11 weeks) accepted the F_1 hybrid skin homograft. Finally, in Group IV in which administration of cells was carried out by both intraperitoneal and intravenous route, 6 out of 7 mice successfully accepted the (A × Z) F_1 hybrid homologous skin graft (Fig. 1). It was interesting to note that the one mouse rejecting the graft in this group was able to retain it for approximately 2 months.

As expected, all Z (C3H) control mice grafted with homologous (A × Z) F_1 hybrid skin rejected this graft in 12 to 15 days after operation (Group V).

Discussion. These results clearly demonstrate that immunological tolerance of homologous skin grafts can be induced in adult inbred mice subjected to repeated administration of viable homologous spleen cells. In our experiments adult Z (C3H) strain mice were used as recipients and F_1 hybrids resulting from cross between A strain females and Z (C3H) males were used as homologous donors of the spleen cells.

This confirms and extends previous reports from this laboratory demonstrating that tolerance of (A × Z) F_1 skin homografts could be established in adult Z (C3H) mice after placing in celomic parabiosis animals of these 2 homologous strains(3). In the latter instance, the essential duration of the parabiotic state to overcome the immunological capacity of the Z (C3H) partner against the (A × Z) F_1 hybrid was approximately 30 days, when permanent tolerance of the F_1 hybrid skin was established.

In the experiments reported here the parabiotic phenomenon was simulated by repeated administration of spleen cells taken from (A × Z) F_1 hybrid donors into adult recipients of the Z (C3H) parent strain. Administration of homologous cells was carried out over periods of one to 11 weeks. The results indicate that a high incidence of tolerance is obtained when cells are administered by both intraperitoneal and intrave-

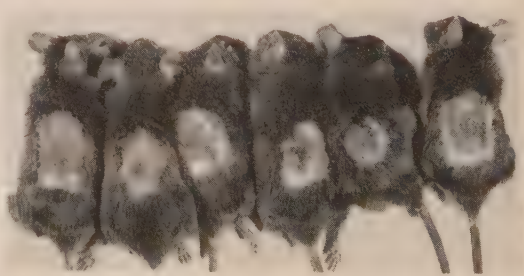

FIG. 1. Z (C3H) strain mice bearing successful homologous skin grafts taken from (A × Z) F_1 hybrid donors. Tolerance induced by repeated intrav. and intraper. administration of (A × Z) F_1 hybrid spleen cells over a period of 7 wk. Picture taken 4½ mo after skin grafting.

nous routes since 6 out of 7 animals thus treated became tolerant and accepted skin grafts taken from homologous donors of the (A × Z) F_1 cross. However, when spleen cells were injected by the intraperitoneal route alone this treatment was not quite sufficient to induce permanent tolerance since only 3 out of a total of 75 Z (C3H) strain animals accepted the F_1 hybrid skin. Even here, however, a suggestion that the immunological barrier was bulged is indicated since none of the non-treated controls accepted F_1 hybrid homologous skin grafts.

Interpretation of these results, as in the case of our studies on tolerance induced by parabiosis, is difficult in the light of current theories regarding immunological tolerance and homotransplantation. In the case of parabiosis we have previously postulated that tolerance of skin homografts induced by this method might be the result of a continuous exchange of all transplantation antigens in the form of intact cells being transferred from one partner to the other in such amount as to inhibit capacity for a homograft reaction. If the parabiosis is maintained for a sufficient length of time this state of nonreactivity could permit establishment of the homologous cells in a form capable of replication in the host thereby maintaining the tolerant state.

By repeatedly injecting homologous spleen cells into adult recipients in a manner designed to simulate as nearly as possible the parabiotic state, tolerance has been obtained in adult animals. The fact that large numbers of spleen cells had to be given during a relatively prolonged period of time combining the intravenous and the intraperitoneal route to achieve tolerance seems to favor this interpretation.

Whatever the final explanation of these findings, it seems clear that immunological tolerance of skin homografts can be induced in adult mice of certain strains submitted to repeated administration of homologous viable spleen cells. It also seems clear that dosage of cells injected, length of time during which treatment has to be maintained and route of administration of cells are also important factors in induction of this phenomenon. These factors, in turn, as in the case of tolerance induced by parabiosis, might also be a function of the antigenic disparity between host and donor.

Summary. 1. Adult mice of the Z (C3H) strain have been made tolerant of skin homografts of the (A × Z) F_1 hybrid strain by repeated intravenous and intraperitoneal injections of homologous spleen cells. 2. Repeated intraperitoneal injections of homologous spleen cells generally failed to induce a tolerant state, although 3/75 recipients accepted the homologous skin homotransplant.

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Effect of the Amount of Tissue Grafted Upon Survival of Skin Homografts.* (26374)

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The effect of the dose or amount of tissue transplanted into homologous hosts upon graft survival has been the subject of several investigations. Medawar(1,2) had previously demonstrated that mean survival time of skin homografts performed in rabbits varies inversely with quantity of tissue grafted, *i.e.*, the smaller the graft the longer the time required for its rejection. These findings were confirmed in rats by Lehrfeld and Taylor(3) and more recently by Zoticov and Budik(4). However, the latter investigators also reported that transplantation of homologous skin in rats in which amount of tissue transplanted was larger than 25 cm^2 was well tolerated and survived for a long time, in some instances up to 8 months following transplantation. The prolonged rejection of large skin homografts observed was interpreted by these workers as the result of the effect of massive dose of foreign antigen contained in the graft leading to a suppression of the immunological capacity of the host.

Stimulated by these findings and because of certain theoretical considerations we decided to reinvestigate this problem determining survival time of male skin isografts of different sizes when placed on isologous female mice known to exhibit graft rejection based on the sex-linked histoincompatibility factor (5). Similar experiments were also performed using several homologous strain of mice. Specifically, this communication reports our observations on this subject and demonstrates that at least in female mice of the C57Bl (Subline 1) strain transplanted with male skin isografts the dose or amount of tissue grafted may be a determining factor in rejection or acceptance of such grafts.

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Method. In one set of experiments, survival of male skin isografts of 2 different sizes when transplanted onto female mice of the C57Bl (Subline 1) strain was studied. One group consisting of 19 female mice of approximately 3 months of age received a 3 cm^2 (1.5 $\times$ 2.0 cm) full-thickness abdominal skin isograft taken from male donors of approximately the same age. The technic of skin grafting has been described(6). Another group of 12 females of similar age received a full-thickness male skin isograft of approximately 24.5 cm^2 (3.5 $\times$ 7.0 cm). The piece of skin to be transplanted was removed from the donor by performing 2 circular incisions comprising skin and subcutaneous tissue, one at the level of the axillae and the other at the level of the iliac crest. After careful dissection of the skin from the adjacent subcutaneous tissue and fat, the tube-shaped graft was pulled apart *in toto* over the head. In placing the graft on the host the skin tube was turned 180° and slipped on over the head of the recipient previously submitted to removal of a similar amount of skin. Interrupted 5-0 silk sutures were used to bring the skin transplant in a close union with the corresponding host tissues. No bandage or dressing was used.

Turning of the graft helped in determining the success or failure of the transplant since the hair in a succesful graft grows in an opposite direction to that on the skin of the host.

Similar experiments were performed using mice of homologous strains in the following donor-host strain combinations, Balb/C $\rightarrow$ DBA (Subline 2), DBA (Subline 2) $\rightarrow$ Balb/C and (A $\times$ Z) $F_1 \rightarrow$ Z.

Following surgery mice were singly housed in plastic mouse cases and fed Purina Laboratory Chow and tap water. Macroscopic inspections of the grafts were performed daily for a period of no less than 5 months.

Results. The results are summarized in

TABLE I. Effect of Amount of Tissue Grafted upon Survival of Skin Homografts and Isografts.

Host strain	Donor strain	Graft size (cm^2)	No. of animals	No. and (%) of animals accepting skin grafts
C57Bl (Sub. 1) ♀	C57Bl (Sub. 1) ♂	3	19	4 (21)
		24.5	12	10 (83)
Balb/C	DBA (Sub. 2)	3	16	0 (0)
		24.5	10	0 (0)
DBA (Sub. 2)	Balb/C	3	20	0 (0)
		24.5	10	0 (0)
Z (C3H)	(A × Z) F_1	3	42	0 (0)
		24.5	6*	—

* All 6 mice died 3 to 5 days after grafting (see text).

Table I. In the group of C57Bl females transplanted with male skin isografts of 3 cm^2 in size, incidence of successful graft acceptance was 21% (4 out of 19). On the other hand, in similar females receiving large male skin isografts (24.5 cm^2) incidence of successful takes was 83% (10 out of 12) (Fig. 1).

In the groups of mice of the Balb/C strain receiving either small or large homologous skin grafts taken from DBA (Subline 2) donors all rejected the skin transplanted. Similar negative results were obtained when DBA (Subline 2) mice were used as recipients and Balb/C as donors regardless of size of the skin transplanted.

FIG. 1. Female mouse of C57Bl (Subline 1) strain bearing a large male skin isograft. Picture taken 5 mo after transplantation.

As expected, small grafts taken from (A × Z)F_1 hybrid donors and transplanted to mice of the Z parental strain failed to survive in all instances. Furthermore, grafting of large grafts in this donor-host strain combination caused death of the host, from 3 to 5 days after operation.

Transfer of tolerance induced by a large skin isograft. Recent experiments from this laboratory have demonstrated that immunological tolerance of male skin isografts induced in female mice of the C57Bl (Subline 1) strain by male antigen exposure at birth could be transferred into adult isologous females by intravenous injection of spleen cells taken from tolerant donors (unpublished). Since large male skin isografts are readily accepted by non-pretreated adult females of this strain thus demonstrating a state of immunological tolerance, it was considered desirable to ascertain whether or not this state of non-reactivity to the male could also be transferred to adult females by injecting these animals with spleen cells taken from donors accepting large male skin isografts. If this were the case it would indicate that the 2 phenomena, *i.e.*, tolerance induced by spleen cell injection at birth and that induced by a direct skin graft had similar underlying mechanisms.

The experiment was carried out as follows: 2 groups of adult female mice of the C57Bl (Subline 1) strain were used as recipients.

TABLE II. Transfer of Tolerance of Male Skin Isografts in Adult C57Bl (Subline 1) Female Mice Injected Intravenously with Spleen Cells Taken from Tolerant Females.

Group	Tolerance of male induced by:	No. of nucleated cells inj. (millions)	No. of inj. females accepting male skin/No. of grafted
I	Inj. of male cells at birth	10-30	8/8 (100%)
II	Large male skin isograft	20-30	0/6 (0%)

One group received an intravenous injection of approximately 20 to 30 million viable spleen cells taken from isologous female donors previously made tolerant of male skin isograft by antigen exposure at birth. Another group received approximately the same amount of cells taken from female donors accepting a large male skin isograft. At 10 to 15 days after treatment both groups of mice received full-thickness abdominal skin isografts taken from adult male donors. The size of the piece of skin grafted was 3 cm^2 (1.5 $\times$ 2.0 cm). After surgery mice were inspected daily for from 3 to 5 months.

The results are listed in Table II. All mice of group I injected with spleen cells taken from females tolerant of male tissue by newborn exposure were susceptible and accepted male skin isografts. In contrast, none of those which had been injected with spleen cells taken from female donors tolerating large male skin isografts were susceptible and these animals readily rejected the male skin isograft. Therefore, it seems clear that while tolerance induced at birth by spleen cell injection is transferable into isologous adult females by inoculating spleen cells taken from tolerant individuals, tolerance resulting from grafting a large piece of male skin is not transferable to another individual by injection of spleen cells.

Discussion. The results of these experiments confirm those previously reported by Zoticov and Budik who found a definite prolongation of skin homografts performed in rats when amount of skin grafted was greater than 25 cm^2. In our experiments this was found to be the case in mice as well. The prolonged survival of the foreign skin graft was, however, evident only in certain donor-host strain combinations such as in female mice of the C57Bl (Subline 1) strain grafted with large male skin isografts. In this regard, while relatively small pieces of skin taken from male donors (3 cm^2) were promptly rejected by the female recipient, those involving approximately 60% of total body surface were accepted in the great majority of cases (83%). In contrast, in other donor-host strain combinations such as Balb/C $\rightarrow$ DBA (Subline 2), DBA (Subline 2) $\rightarrow$ Balb/C or (A $\times$ Z)F_1 $\rightarrow$ Z (C3H) both small and large skin homografts were rejected in all instances.

It is interesting to note that in the combination of (A $\times$ Z)F_1 hybrid donor and Z(C3H) hosts, grafting of the large piece of homologous F_1 hybrid skin resulted in early death of the Z strain recipient. The Russian workers have also mentioned early death in a high proportion of their rats receiving large skin homografts. As in the case of the rats the early mortality observed in our group of Z strain mice receiving (A $\times$ Z)F_1 hybrid skin grafts cannot be explained on the basis of trauma and surgical manipulation since large skin isografts performed in a small group of Z strain mice resulted in acceptance of the graft in all instances without either early or delayed mortality of the host. Furthermore, no mortality due to grafting was observed in either Balb/C strain mice receiving grafts taken from DBA (Subline 2) donors, in DBA (Subline 2) transplanted with skin taken from Balb/C homologous donors or in C57Bl (Subline 1) females receiving large skin isografts taken from male donors.

The early mortality observed in Z strain animals receiving large (A $\times$ Z) F_1 hybrid skin homograft and the absence of ill effects in the other donor-host strain combinations used is difficult of interpretation. However, one might tentatively postulate that this phenomenon is related to degree of antigenic disparity between donor and host, which was slight in case of male and female mice of the C57Bl (Subline 1) or Balb/C and DBA (Subline 2) strain and stronger between (A

$\times$ Z) F_1 and Z strain mice.

Recently Trentin(7) has attributed the early mortality obtained in sublethally irradiated mice receiving homologous bone marrow transplants to a strong rejection reaction on the part of the host against the grafted cells. In our experiments a similar mechanism might account for the early death observed in Z mice grafted with large (A $\times$ Z) F_1 homologous graft. The weakness of the antigenic disparity between male and female mice of the C57Bl (Subline 1) strain and between DBA (Subline 2) and BALB/C strain mice might preclude a rejection reaction strong enough to result in death of the host. Thus, it is postulated that the death of Z mice receiving very large (A $\times$ Z)F_1 skin homograft is due to the massive rejection reaction itself.

The finding that large male skin isografts (24.5 cm^2) are accepted by females of the C57Bl (Subline 1) stock is of particular interest. In all respects the inability of the female to reject such an amount of male skin cannot be distinguished from the situation which pertains in classical immunological tolerance induced by administration of male spleen cells at birth or during adult life. In both instances the male graft is established permanently. However, the underlying mechanisms of both types of tolerance might be entirely different. In this regard, Zoticov and Budik have postulated that acceptance of large skin homografts in rats might be the result of the effect of a massive dose of foreign antigen probably contained in the grafts which leads to suppression of the immunological capacity of the host. Tolerance induced by administration of male spleen cells at birth might also result from the presence of amount of antigen which overwhelms the immunological capacity of the host, with the requirement for antigen being considerably smaller under circumstances in which the animals' total complement of immunologically competent cells is low (as in the neonatal period or following irradiation). Following spleen cell injection it is presumed that the tolerant state is maintained by release of foreign antigen by the foreign cells in residence in the recipient tolerant host. This possibility is supported by the observation that classical tolerance to a certain strain induced with spleen cells from that strain can be transferred to a new host by injection of spleen cells from the recipient tolerant animal(8). Thus the site of replication and release of the antigenic components is assumed to reside in the chimeric spleen and possibly other reticulo-endothelial tissues. It is consequently of great interest that the tolerant state induced with massive-sized skin homografts cannot be transferred by injection of spleen cells. It is presumed in this instance that the tolerant state may be induced by massive antigenic exposure from the huge skin graft and that the tolerant state is maintained by constant delivery of antigen from the skin graft itself.

It seems clear, therefore, that tolerance of male skin isograft in adult female mice can be induced by grafting the females with a male skin graft involving approximately 60% of total body surface. The antigenic disparity between host and donor as well as total amount of foreign antigen to which the host is exposed, which actually may be a function of the former, appear to be of importance in suppressing the immunological capacity of the host to react against the specific foreign antigens.

Summary. 1. Female mice of the C57Bl (Subline 1) strain capable of rejection of small male skin isografts regularly accepted large male skin isografts. 2. Large homologous skin grafts between genetically closely related strains were regularly rejected as were small skin homografts. 3. Large skin homografts between strains of mice characterized by strong histocompatibility differences regularly resulted in early death of the recipient. 4. Tolerance of C57Bl (Subline 1) female mice to male skin isografts induced by spleen cell injection could be transferred by injection of spleen cells from tolerant donors to new females during the post-neonatal period. Attempts to transfer tolerance induced with a large skin graft were unsuccessful.

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Male Skin Isograft Survival in Pregnant and Multiparous Female Mice.* (26375)

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Breyere and Barrett have demonstrated that multiparous female mice of the BALB/cAn strain which had borne litters of strain DBA/2 males became tolerant of a transplanted sarcoma originated in DBA/2 strain mice(1). Recently Prehn(2) demonstrated that female mice of the C57Bl/An strain parous by repeated matings to littermate males accepted male skin isografts in high proportion of cases. Furthermore, the incidence of tolerance of male graft was proportional to the number of litters borne by the female recipient. Thus, while 60% of the females having 1-3 litters were tolerant of male skin isografts, 90% of those having 4-6 litters were also tolerant and accepted male skin isografts.

In the experiments reported herein studies were undertaken to determine male skin isograft survival in multiparous female mice of the C57Bl (Subline 1)§ strain. Female mice of this strain regularly reject skin grafts taken from isologous males although the antigenic disparity between donor and recipient is rather weak allowing induction of tolerance in the females to male skin graft by exposure to the male antigen during the neonatal period as well as during adult life(3,4).

The experiment was carried out as follows: Female breeder mice in the C57Bl (Subline 1) inbred colony were separated from the breeder boxes after they had borne 2-6 litters (average 3.5). One group of 16 animals received a full-thickness skin graft (1.5 × 2.0 cm in size) taken from isologous males. Grafting was performed at midpregnancy (12-14 days after fertile mating). Another group of 11 mice received the male skin graft 9-16 days after delivery of last litter. A third group of 9 mice received the male skin graft 43-105 days after delivery of last litter. Finally, a fourth group consisted of 19 virgin controls receiving male skin isografts.

The technic of skin grafting as well as the criteria used to judge the success or failure of the graft were the same as previously described(5). Mice were singly housed in plastic boxes and fed Laboratory chow and tap water.

The results are listed in the Table. There was only a slight increase in male graft acceptance in the females receiving the transplant either during pregnancy or 9-16 days after delivery as compared to the virgin control group. However this difference is not statistically significant. In addition, no significant differences in rejection time of the

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§ Mice of this strain were separated from Dr. J. J. Bittner's mouse colony in 1956 and maintained in our laboratory by rigorous inbreeding.

TABLE I. Survival of Male Skin Isografts in Pregnant and Parous Female Mice of C57Bl (Subline 1) Strain.

Group	No. of mice	No. of previous pregnancies (mean)	Time of grafting	No. and (%) of female mice accepting male skin grafts
I	16	3.6	Mid-preg.	5/16 (27.5)
II	11	3.5	9-16 days after deliv.	3/11 (27)
III	9	3.4	43-105 days after deliv.	2/9 (22)
IV	19	0	—	4/19 (21)

grafts were observed, this being approximately 8 to 12 days in all groups.

It seems, therefore, that multiparous mice of the C57Bl (Subline 1) do not show a significant increase in male isograft acceptance when compared to virgin controls. Our results seem in conflict with those recently reported by Prehn. However, this could be perhaps explained on the basis of a different genetic make up of our C57Bl (Subline 1) strain mice as compared to those of the C57 Bl/An which Prehn has used.

Summary. Experiments were undertaken to determine male skin isograft survival in multiparous female mice of the C57Bl (Subline 1) strain, as compared to virgin controls. No significant difference in male isograft acceptance was found in multiparous mice as compared to that in virgins.

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Alteration of Susceptibility of Embryonated Eggs to Newcastle Disease Virus by *Escherichia coli* and Endotoxin. (26376)

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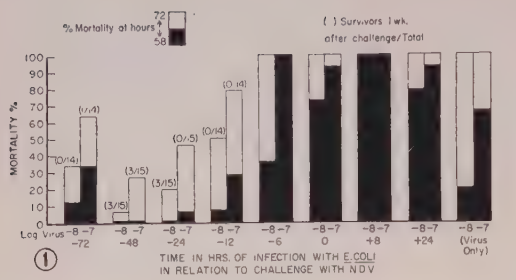
It was recently demonstrated(1) that infection with *Escherichia coli* markedly increased resistance of chick embryos to subsequent challenge with *Vibrio cholerae*. Resistance could also be stimulated by inoculation of killed *E. coli* cells or endotoxin preparations prior to challenge. Thus, it seems apparent that the chick embryo is capable of a response to endotoxin, similar to that described in mature animals(2,3), which results in an enhanced non-specific resistance to bacterial infection. It is of obvious importance, then, to determine whether this enhanced resistance extends to virus infection as well. Newcastle disease virus was selected as the agent for test since it is stable and lethal for chick embryos in low dosage, infection can readily be proven by hemagglutination, and it has been reported on a number of occasions(4) to be inhibited by host serum factors which may play roles in natural resistance.

Materials and methods. Antibiotic-free, pullorum clean White Leghorn chick embryos were used. Eggs from a single flock were delivered at a specified age, usually 10 days, and were incubated on arrival with the air sac end up at a slight angle on racks in a humidified egg incubator at 36-37°C. Methodology was similar to the previous study(1). Embryos were challenged allantoically with 0.1 ml of virus dilution on day 13 through a small hole punched in the shell. At least 15 eggs were used per group within an experiment. Viability of embryos was determined by candling using criteria of well defined blood vessels and embryonic movement. *Escherichia coli* strain 9, serotype 0111:B4, and



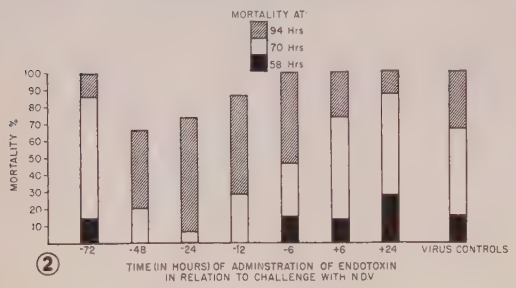


FIG. 1. Effect of *E. coli* on mortality of chick embryos challenged with N.D.V.

FIG. 2. Effect of endotoxin on mortality of chick embryos challenged with N.D.V. (10^{-8} dil'n).

E. coli endotoxin, preparation #36, described previously(1) as being among several strains and endotoxin preparations which elicit the protective response against *V. cholerae* infection, were used in this study. Neither endotoxin nor *E. coli* strain 9 administered allantoically in the dosages indicated was lethal for chick embryos to any significant extent. The Newcastle disease virus (NDV) strain AMS2, was obtained from the Dept. of Virology of this Institute. For stock virus, 10-day-old eggs were inoculated with a 1:1000 dilution. The allantoic fluid was harvested aseptically at 48 hours, centrifuged and stored in vials at –20°C. This stock proved to be quite stable; over a 9 month period, 0.1 ml doses of freshly prepared 10^{-8} dilutions in saline killed approximately 50% of 13 day embryos within 3 to 4 days.

Results. Effect of E. coli on mortality of chick embryos challenged with NDV. Groups of eggs were inoculated allantoically with *E. coli* at varying intervals before and after virus challenge. The *E. coli* inocula, prepared by suspending and diluting with sterile saline the 18-hour growth from a heavily streaked meat extract agar Petri dish, ranged between 1500 and 4600 viable cells. Previous experience(1) had shown that these cells multiply to a population of approximately 10^8 per ml of egg within 24 hours. Eggs were challenged on day 13 with 0.1 ml of 10^{-6} or 10^{-7} dilutions (approximately 100 and 10 LD_{50}) of stock NDV in saline. Since deaths from the virus infection begin to occur by 48 hours post challenge in controls, the occasional eggs which succumbed by 24 hours after virus challenge were discarded. Eggs were candled again at 58 and 72 hours and results plotted in Fig. 1. *E. coli* infection had marked effects on lethality of NDV. Some evidence of protection was noted in eggs inoculated with *E. coli* 72 hours prior to NDV. It was most marked when *E. coli* cells were administered 48 hours prior to virus and the protective effect was still present at 24 and 12 hours. When the *E. coli* cells were inoculated 6 hours before virus or later, the effect was rather that of virus enhancement. Seventy-two hours after virus inoculation, all the control eggs and those inoculated with *E. coli* at –6, 0, +8, and +24 hours were dead. Mortality of embryos in the other groups increased by 72 hours in proportion to the virus inoculum and to the 58 hour mortality. Deaths continued to occur throughout the incubation period of these embryos, but at the embryonic age of 20 days there were still a few survivors some of which had pipped through their shells. The survivors were discarded at this time. Allantoic fluid of eggs dying late was found to contain hemagglutinin for chick and for human O erythrocytes which was specifically inhibited to high titer by NDV antiserum. Mortality was not affected by inoculation of saline 48 hours prior to or 6 hours after virus in groups of control eggs.

Effect of endotoxin on mortality of embryos challenged with NDV. In a similar manner, groups of eggs were inoculated allantoically at intervals with 4 μg of endotoxin (0.1 ml of a saline solution of 40 μg of endotoxin per ml) and challenged with NDV. Results (Fig. 2) were similar to those obtained in eggs infected with living *E. coli* in that there were phases of increased resistance and susceptibility. Neither effect, however, was of the magnitude observed when living *E. coli*

were used. Protection until time of hatching was not achieved in this experiment although occasional endotoxin treated eggs survived 5 days and possibly longer following virus inoculation. Living *E. coli* might be more effective since they could provide a continuing source of endotoxin. In this, and other experiments, greatest survival and also greatest mortality are obtained with correspondingly later injections of endotoxin than of viable *E. coli*. Thus, a period approximately 24 hours prior to virus appears to be the most effective time to administer endotoxin for maximal protection while −48 hours appears to be optimal in this regard when living *E. coli* are used. Similarly, enhancement of mortality is most effectively demonstrated with administration of endotoxin 24 hours after virus as opposed to +8 hours with viable *E. coli*.

Discussion. Infection of chick embryos with *E. coli* has marked effects on their susceptibility to challenge with bacterial(1) or viral pathogens. In both instances, the effects are duplicated by injection of purified endotoxin preparations at intervals which correspond to the time one would anticipate endotoxin to become available from the *E. coli* infection. Thus, it may be concluded with fair degree of certainty that the effects of *E. coli* infection may be attributed to endotoxin. In the case of NDV infection, phases of both enhanced resistance and susceptibility are observed dependent on time of administration of endotoxin or initiation of infection with *E. coli*.

Similar effects on resistance to bacterial infection in mice have been obtained by pretreatment with endotoxin(2,3) and recently reports have appeared on alteration of resistance to viral infection as well. Xerosin, an endotoxin like, heat-stable, acid-precipitable material from *Achromobacter xerosis,* has been demonstrated to suppress the neurotoxic effect of influenza virus in mice when given intracerebrally (IC) before, but not after, inoculation of virus by the same route(5). If injection of xerosin was delayed until 24 hours after virus, it was not only ineffective, but appeared to make the situation worse (enhance the neurotoxic effect). Earlier(6), this material had been tested for antiviral activity against influenza A virus in chick embryos. In tests in which xerosin was administered 1 hour before, 1 hour after, or simultaneously with virus it effected a 2-fold reduction in formation of viral hemagglutinin, but did not reduce infective titer. Possibly more striking results could have been obtained using other time intervals. Groupé *et al.*(5,7) also stated that a preparation of receptor destroying enzyme (RDE) of *Vibrio cholerae,* which was reported by Wagner(8) to protect mice against influenza neurotoxin, could be heated to 120°C and still retain protective activity. This treatment completely inactivated RDE activity of the preparation which is essentially a culture filtrate and undoubtedly contains some cholera endotoxin. This view was taken by Wagner, *et al.*(9), who reviewed the earlier literature on this subject and similarly attributed activity of xerosin to its endotoxin content.

The latter workers were able to increase resistance of mice to eastern equine encephalitis and encephalomyocarditis viruses by administration of endotoxin. Endotoxin had no effect on viral infectivity for cell cultures, nor could an antiviral substance be demonstrated in brains of mice made resistant by IC injection of endotoxin, although there was evidence of reduced virus content in brains of protected mice. Hook and Wagner(10) were also able by prior treatment with endotoxin to increase resistance of mice to neurotoxicity of influenza virus, but not to intracerebral infection with neurotropic influenza virus or lymphocytic choriomeningitis virus. Gledhill(11) found that endotoxin delayed death of mice following large doses of ectromelia virus. Similar effects were obtained with saccharated iron oxide and the author concluded that stimulation of the reticulo-endothelial system (RES) was the mechanism involved. Additional evidence for the role of RES in defense against virus infection is derived from the reduced blood clearance of NDV in thorotrast treated mice (12). The role of inflammatory cells was further implicated by the observations of Kil-

bourne(13) that survival of chick embryos infected with influenza B virus may be prolonged or shortened depending on time of injection of cortisone which was shown to suppress inflammatory response.

In our study, the protective effect is manifested primarily in a limited prolongation of survival time although occasional embryos will survive to time of hatching. Living *E. coli* seem to be more effective in this regard than endotoxin. Preliminary observations based on hemagglutinin titrations and infectivity determinations of allantoic fluid from protected and control eggs indicate that the protective effect is associated with a temporary suppression of virus levels in allantoic fluid. Therefore, whatever mechanisms may be involved in the widely demonstrated endotoxin provoked alterations of resistance, they are available, at least in part, to the developing chick embryo which should be a valuable tool in further study of these phenomena.

Summary. Phases of enhanced resistance and susceptibility to Newcastle disease virus were induced in chick embryos by infection with *E. coli*. These effects were duplicated by treatment of embryos with *E. coli* endotoxin at appropriate times.

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Effects of Various Amino Acids Upon Glucose Uptake of Rat Epididymal Adipose Tissue.* (26377)

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Insulin-like activity, as measured by glucose uptake of the rat diaphragm, has been stated to be increased by addition of the amino acids, hydroxyproline and lysine, in low concentrations to the incubation medium (1). Insulin-like or anti-insulin effects of various amino acids were measured in a bio-assay system utilizing glucose uptake by Wistar rat epididymal adipose tissue(2). This bio-assay method has been shown to be sensitive to as little as 1-10 microunits insulin/ml.

Methods. This method of insulin bio-assay has been described and analyzed in detail previously(3). In this study, 35 to 60 mg segments of epididymal adipose tissue

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TABLE I. Relationship of Amino Acids at 10^{-3} M Concentration to Glucose Uptake.

Amino acid	No. of determinations	Difference in glucose uptake (mg glucose/g fat) Mean	S.E.M. (±)	Significance of amino acid mean uptake compared with control mean uptake, P
Arginine	9	−1.47	.221	<.001
Glutamic acid	10	− .94	.37	>.02, <.05
Histidine	9	−1.16	.32	<.01
Isoleucine	10	− .94	.51	>.05, <.10
Leucine	72	− .77	.28	<.01
Lysine	25	+ .22	.45	>.6
Proline	10	− .59	.31	>.05, <.10
Tyrosine	5	−2.53	.23	<.001

were excised from 100-180 g male Wistar rats. The tissue was added to beakers containing 2.5 ml of Krebs-bicarbonate buffer, glucose, 0.2% serum albumin, and L-amino acids or insulin. In some instances an L-amino acid and insulin were added simultaneously. Non-insulin controls were present in each set of determinations. Incubation proceeded in a Dubnoff metabolic shaking incubator for 5 hours, at 36.5°C, under 95% CO_2. After deproteinization by the Somogyi method, residual glucose was determined on 1 ml aliquots of incubating medium by the Glucostat method. Glucose uptake was calculated as mg of glucose per g of adipose tissue. Various concentrations of insulin and amino acids, individually or in combination, were tested this way and compared with appropriate controls.

The differences between glucose uptake in the presence of an amino acid and the corresponding mean control uptake represent effective glucose uptake of the individual amino acid. These results, calculated as mean difference of uptake, are expressed as milligrams of glucose per gram of fat tissue. Statistical significance of these differences were ascertained with the Student "t" method.

Results and discussion. The amino acids tested at 10^{-2}–10^{-3} M concentration exhibited inhibition of glucose uptake by the fat pad. Six amino acids exhibited statistically significant inhibition of glucose uptake. These were arginine, glutamic acid, histidine, leucine, lysine, and tyrosine. Two amino acids, isoleucine and proline, exhibited slight inhibition at 10^{-3} M concentration, but this was statistically not significant (Table I).

In all determinations involving more than one concentration of the amino acid tested, it was invariably found that a decrease in amino acid concentration diminishes the inhibitory action of the amino acid (Table II).

TABLE II. Relationship of Dilution of Amino Acid to Glucose Uptake.

Amino acid	No. of determinations	Difference in glucose uptake (mg glucose/g fat) Mean	S.E.M. (±)	Significance of amino acid mean uptake compared with control mean uptake, P
10^{-2} M Glutamic acid	10	−2.16	.42	<.001
10^{-3} M " "	10	− .94	.37	>.02, <.05
10^{-3} M Isoleucine	10	− .94	.51	>.05, <.10
10^{-7} M "	4	− .34	.39	>.4 , <.5
10^{-2} M Leucine	15	−2.68	.55	<.001
10^{-3} M "	72	− .77	.28	<.01
10^{-5} M "	32	+ .49	.33	>.1 , <.2
10^{-7} M "	28	+ .53	.27	>.05, <.10
10^{-2} M Lysine	15	−1.91	.53	<.01
10^{-3} M "	25	+ .22	.45	>.6

Preliminary observations were made utilizing a combination of leucine, at 10^{-3} M concentration, and insulin at 100 micro-units/ml. Coincidently, separate determinations for leucine at 10^{-3} M concentration and insulin at 100 micro-units were obtained. Individually, there was a significant increase in uptake with insulin and a signfiicant decrease in uptake with leucine. The combination of insulin and leucine demonstrated a significant increase in glucose uptake above the non-insulin control while being associated with a significant decrease in glucose uptake as compared with the insulin control. In 21 determinations, the mean difference in glucose uptake of the insulin-leucine combination compared with the non-insulin control was + 2.58 mg glucose/g adipose tissue ($p<.001$). When the combination was compared with the insulin control, the mean difference in glucose uptake was –2.04 mg glucose/g adipose tissue ($p<.001$).

A pH effect was considered as a possible factor in the inhibitory action of leucine upon glucose uptake. Determinations of pH performed upon leucine samples were identical with those of control samples, thereby eliminating pH changes as significantly related to the inhibitory action of leucine.

Previous unpublished studies, utilizing an incubation time of 2 hours, demonstrated statistically significant inhibition of glucose uptake by adipose tissue in the presence of certain amino acids. The magnitude of the effects tended to be less than appeared in the present study utilizing longer incubation times.

Summary. Six of 8 amino acids tested at concentrations of 10^{-2}–10^{-3} M demonstrated significant inhibition of glucose uptake by adipose tissue. This inhibitory action was diminished as amino acid concentration was further decreased.

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The Value of Certain Steroidal Sapogenins in Rations of Fattening Lambs and Cattle. (26378)

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(Introduced by A. R. English)
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The growth-promoting action for ruminants of diethylstilbestrol and certain other estrogens is well known. However, because of the estrogenic activity of these compounds, there must be close control of the quantities given in practical feeding operations. Combinations of estrogens with androgens or progesterone have been used as growth stimulants, the estrogenic action being diminished by the other hormone so that anabolic action predominates. However, it would be desirable to have available a single, non-estrogenic compound with substantial anabolic action (1).

There are structural similarities between natural animal steroid hormones and certain plant steroids. These plant products are not known to have estrogenic activity, nor have they been evaluated for effect on ruminant productive performance. The present report provides data on growth rate, feed efficiency and carcass quality of fattening steers and lambs fed plant steroidal sapogenins, saponins, and a natural source of unisolated sapogenins. The products tested were smilagenin and smilagenin saponin isolated from *Agave lecheguilla,* ground *Agave lecheguilla*

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TABLE I. Experimental Rations.

	Cattle	Lambs*
Cracked shelled corn	Full fed	
Alfalfa hay/day, lb	3	
Supplement/day, lb	2	
Composition of supplement or complete pellet		
Ground yellow corn		55
Sun-cured alfalfa meal		35
Soybean oil meal	69.05	
Dehydrated alfalfa meal	10.00	
Liquid molasses		5
Dried "	10.00	
Urea	5.00	1
Bone meal		1
Dicalcium phosphate	4.50	
Ground limestone	1.00	
Trace mineral premix	.40	
Vitamin A†	.05	
Bentonite		3
	100.00	100

* The lamb ration was made into 3/8 inch pellets and self-fed.

† Gelatin-stabilized vit. A mix containing 10,000 U.S.P. units of vit. A palmitate/g.

containing smilagenin in the form of a saponin, and sarsasapogenin, tigogenin, diosgenin and hecogenin from commercial sources.

Procedure. Five lamb experiments were conducted with graded levels of smilagenin ranging from 2 to 24 g per ton of total ration. Lamb experiment 3 included a group receiving ground *Agave lecheguilla* (1.6% smilagenin) supplying the equivalent of 8 g smilagenin per ton of ration. Lamb experiment 4 included groups receiving sarsasapogenin, diosgenin and tigogenin at 8 g per ton of ration. Lamb experiment 5 included groups receiving smilagenin saponin at levels of 4, 8 and 16 g per ton, hecogenin at 8 g per ton and diethylstilbestrol at 1.25 g per ton.

In all lamb experiments, 12 animals were allotted per treatment, the controls comprising 2 replicate lots of 12 each. Individual weighings were made every 14 days. Some death losses occurred and the data have been corrected for these losses.

In cattle experiment 1, smilagenin at 20 mg and 100 mg per head was compared with oral stilbestrol at 10 mg per head. In cattle experiment 2, smilagenin at 20 mg per head was compared with stilbestrol at 10 mg per head. The steers were weighed at 28-day intervals. Basal rations for the lamb and cattle trials are presented in Table I.

Results. Weight gains, feed efficiency, dressing per cent and carcass grade data for the lambs are presented in Table II. In Exp. 1, the 4 g of smilagenin per ton of ration had no effect upon weight gains or feed efficiency, but dressing per cent and carcass grade were improved. In Exp. 2 both the 2 and 8 g levels of smilagenin improved weight gains and feed efficiency. The 8 g level exerted a marked effect, bringing about a 32% increase in gain and a 31% improvement in feed efficiency. Carcass grade was significantly improved by both levels of smilagenin, but there was no effect upon dressing per cent. In Exp. 3, no response was noted to the 2 g level of smilagenin, but the 8 and 24 g levels increased gains, the increase being 11% with 24 g. Dressing per cent and grade were not affected. The ground *Agave* plant produced weight gain increases similar to those obtained with 8 g of smilagenin.

In Exp. 4, significant improvements in rate of gain occurred with 4, 8 and 24 g of smilagenin per ton. The 8 g level brought about a 34% increase over the control and a 17% improvement in feed efficiency. The two lower levels of smilagenin improved both dressing per cent and carcass grade. The 8 g of sarsasapogenin produced gains almost identical with those obtained from 8 g of smilagenin. Dressing percent and carcass grades were generally similar to those obtained with the smilagenin treatments. Diosgenin and tigogenin at 8 g per ton did not improve gains over the control and these compounds appeared to be toxic in the early part of the experiment as indicated by feed intake and weight gains.

In Exp. 5 the 4 g and 16 g levels of smilagenin produced comparable improvements in growth, feed efficiency and grade. The 8 g treatment group was not appreciably better than the basal; however it was noted that this supplemented group included one very poor-performing lamb. If this lamb had been eliminated from the data, performance of the 8 g group would have been comparable to that of the other 2 smilagenin-fed

TABLE II. Response of Fattening Lambs (Wethers) to Various Plant Steroids.

Treatment	No. lambs	Avg initial wt, lb	Avg daily gain, lb	Lb feed per lb gain	Dressing, %†	Carcass grade‡
		Exp. 1—53 days				
Basal	23	86.5	.688	6.50	46.81	8.49
+ Smilagenin, 4 g/ton	12	84.7	.633	7.48	48.01	9.17
		Exp. 2—56 days				
Basal	23	70.3	.344	10.38	49.60	8.61
+ Smilagenin, 2 g/ton	10	68.5	.384	8.21	49.46	9.20*
+ " , 8 "	11	69.4	.455	7.14	49.09	9.27*
		Exp. 3—56 days				
Basal	23	71.0	.368	6.84	50.02§	9.82
+ Smilagenin, 2 g/ton	12	71.4	.345	7.36	— §	—
+ " , 8 "	12	69.2	.390	6.23	50.03	9.25
+ " , 24 "	12	69.3	.409	6.11	50.47	9.91
+ Gr. *Agave lechequilla* equiv. to 8 g/smilagenin/ton	12	71.0	.385	7.40	49.98	9.36
		Exp. 4—68 days				
Basal	21	74.0	.320	9.29	44.98	6.84
+ Smilagenin, 4 g/ton	11	73.4	.398*	8.52	45.60	7.82
+ " , 8 "	12	74.6	.430*	7.69	45.54	7.67
+ " , 24 "	12	74.0	.347*	9.16	45.07	6.83
+ Sarsasapogenin, 8 g/ton	12	74.2	.428*	8.29	45.80	7.50
+ Diosgenin, 8 "	11	75.9	.322	9.28	44.48	7.00
+ Tigogenin, 8 "	10	77.4	.304	9.98	44.91	6.90
		Exp. 5—70 days				
Basal	23	65.1	.392	7.38	— ‖	6.80‖
+ Smilagenin, 4 g/ton	11	65.2	.443	7.27	—	8.00
+ " , 8 "	11	66.2	.402	7.35	—	6.75
+ " , 16 "	12	66.7	.440	7.29	—	9.50
+ Smilagenin saponin, 4 g/ton	11	65.8	.403	7.22	—	7.46
+ " " , 8 "	12	64.0	.384	7.51	—	6.75
+ " " , 16 "	12	65.4	.379	7.63	—	6.75
Hecogenin, 8 g/ton	11	66.0	.429	7.23	—	7.46
Diethylstilbestrol, 1.25 g/ton	12	68.0	.379	8.05	—	7.75

* Significant over controls (P = 0.05).

† In Exp. 1 and 3 dressing % based on cold carcass weights and in Exp. 2 and 4 based on warm carcass weights.

‡ 10 = low choice, 9 = high good, 8 = avg good.

§ In the basal groups carcass grade and dressing % available on 12 animals only as 11 were retained for additional experimental work. All 12 lambs on the low level of smilagenin were also retained.

‖ Dressing % figures were not available in Exp. 5 and grade values were based on live grade evaluation.

groups. Smilagenin saponin at the 4 g level produced only slightly better performance than the control and the consistently depressed performance obtained with each increment suggests that the saponin form of steroidal sapogenins is not well tolerated. Hecogenin at 8 g produced improvements in growth and feed efficiency comparable to those with the several levels of smilagenin. Stilbestrol did not enhance performance in this experiment.

Results of the 2 cattle experiments are presented in Table III. In the first, an increase of 0.27 lb in average daily gain followed feeding of 20 mg smilagenin and an increase of 0.15 lb followed 10 mg of stilbestrol. Feed efficiency was improved by 20 mg of smilagenin and dressing per cent was improved by both levels of smilagenin and by stilbestrol. Carcass grade appeared to be improved by stilbestrol.

In cattle experiment 2, average daily gains were increased 0.17 lb by 20 mg smilagenin and 0.31 lb by 10 mg stilbestrol. Feed efficiencies were increased by both compounds. Dressing per cent appeared to be lowered by the diethylstilbestrol. Carcass grade was improved markedly by the smilagenin and a

TABLE III. Response of Fattening Steers to Smilagenin and Diethylstilbestrol.

Treatment	No. steers	Avg initial wt, lb	Avg daily gain, lb	Lb feed per lb gain	Dressing, %*	Carcass grade†
		Exp. 1—98 days				
Basal	9	924.6	2.14	12.02	60.67	10.00
Smilagenin, 20 mg/head	8	951.5	2.41	11.44	61.67	9.88
" , 100 "	8	941.6	2.21	12.29	61.98	9.88
Diethylstilbestrol, 10 mg/head	8	920.5	2.29	11.73	61.92	10.62
		Exp. 2—139 days				
Basal	8	677.1	2.57	9.56	57.28	7.88
Smilagenin, 20 mg/head	8	680.1	2.74	8.68	57.14	8.75
Diethylstilbestrol, 10 mg/head	8	674.8	2.88	8.88	56.60	8.25

* Based on cold carcass wt. † 10 = low choice, 9 = high good.

slight improvement was noted in the stilbestrol group.

Discussion. In the lamb trials, the 8 g level of smilagenin appeared to be most consistent in increasing weight gains. The average increase obtained with this level in 4 trials was 17.7% above controls and average feed efficiency increase was 16.2%. This level supplied approximately 12 mg smilagenin per lamb per day. Nearly identical growth responses (34%) were obtained with 8 g levels of sarsasapogenin and smilagenin (one comparison-experiment 4). In general, sarsasapogenin appeared to increase dressing per cent and improve grade. Hecogenin was comparable to smilagenin in improving growth (Exp. 5) and appeared to improve feed efficiencies and grade. Diosgenin and tigogenin did not improve performance (Exp. 4).

Measurement of the bulbo-urethral glands in lamb experiments 3 and 4 did not reveal an enlargement from plant steroid feeding as is noted with lambs fed diethylstilbestrol(1). Mouse uterine assays in this laboratory did not indicate estrogenic activity in smilagenin.

In the 2 cattle experiments the 20 mg daily feeding level of smilagenin improved gain by an average of 9.6% as compared with 9.5% for 10 mg stilbestrol.

No basis is immediately available to explain the mechanisms whereby smilagenin, sarsasapogenin or hecogenin bring about more rapid gains. Smilagenin and sarsasapogen are related in that smilagenin can be obtained by acid isomerization of sarsasapogenin(2). The mechanism of the growth-stimulating action of the plant steroids may be due to an anabolic action similar to that of stilbestrol but wtihout the undesirable estrogenic effects.

These studies indicate that smilagenin and certain other plant steroids may be of practical use in production of fat lambs and steers. Additional study is needed before the full significance of these findings can be assessed.

Summary. 1. Smilagenin fed to lambs at levels of 2, 4, 8 and 24 g per ton of complete feed improved growth rate and feed efficiency. The most consistent results were obtained with 8 g per ton, or approximately 12 mg per lamb per day. Dressing per cent and carcass grade tended to be improved by addition of smilagenin. Ground *Agave lecheguilla* containing smilagenin in the saponin form appeared to be quantitatively similar to the free sapogenin; however higher levels of the isolated saponin depressed performance. Sarsasapogenin or hecogenin at 8 g per ton in separate tests exerted a growth effect comparable to that from smilagenin. Diosgenin and tigogenin were not beneficial under conditions tested. 2. In 2 trials, steers fed 20 mg of smilagenin daily showed weight gains similar to those of animals receiving 10 mg stilbestrol daily. In one trial, 100 mg of smilagenin was not as effective as 20 mg.

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Distribution and Excretion of Heparin. (26379)

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The mechanisms responsible for the rapid termination of anticoagulant action of intravenously administered heparin have not been well established. A single average dose of 0.5 to 1.0 mg/kg given to mammals produces almost immediate anticoagulant effect of 2 to 4 hour duration. Increasing the dose increases the intensity of the effect but does not proportionately increase its duration. Excretion of heparin in the urine during the period of systemic anticoagulant effect does not appear to account for termination of effect of the drug. A heparin inactivating enzyme "heparinase" has been described in liver extracts but recent studies indicate that this may not be a true enzyme. Rather, Loomis(1) has obtained *in vivo* evidence which indicates that liver is not selectively involved in terminating the action of heparin. Therefore, although a "normal" blood concentration of endogenous heparin has been described(2,3) it appears that exogenous heparin is either degraded or stored in inactive forms and is subsequently slowly excreted from the body. This study is concerned with some possible mechanisms responsible for terminating the action of administered heparin.

Methods. Sodium heparin S^{35} was biologically synthesized. This consisted of administering $Na_2S^{35}O_4$ (0.5 millicurie, intravenous) once daily on 2 successive days to each of 2 large dogs. Forty-eight hours after the initial dose, the animals were sacrificed, the livers were removed, combined, homogenized with water and extracted exactly according to the method of Eiber and Danishefsky(4). No carrier heparin was added during the extraction procedure. The final product obtained from 1250 g of liver consisted of 132 mg of a light brown powder having 6300 cpm/mg. The product was free of inorganic sulfur and was electrophoretically homogenous. *In vitro* assay showed 72 U.S.P. anticoagulant units per mg and metachromatic assay (with toluidine blue) showed an activity of 85 units per mg. This product hereafter is referred to as heparin S^{35}.

Three small (5 to 6 kg) dogs were anesthetized with sodium pentobarbital (30 mg/kg, intravenous). A section of a femoral vein was exposed and the urinary bladder was cannulated. Each dog was given heparin S^{35} (3 mg/kg, intravenous) and intensity and duration of anticoagulant effect was determined according to a modified Lee-White 3-tube clotting time method(5). Plasma and urine isotope activity was determined on samples obtained at 15 minute intervals for 2 hours by the method described by Walkenstein(6).

Twenty rats were injected with heparin S^{35} (20 mg/kg, intravenous). Groups of 4 of these animals were sacrificed at 2, 6, 12, 18 and 24 hours post injection. Three animals in each group were sacrificed by cervical vertebra crush, and the fourth animal was etherized and prepared for obtaining peritoneal mast cell suspensions.

Samples of plasma, blood cells, kidney, liver, spleen, heart, lungs, small intestine, skin and skeletal muscle were digested in concentrated nitric acid and isotope activity of the product of digestion was measured. The counts were corrected for background activity and converted to mg of heparin S^{35}.

The mast cells in the peritoneal washings were separated by the sucrose differential specific gravity method of Glick *et al.*(7). A suspension containing nearly 100% mast cells was thereby obtained. Radioautographs were made from smears of the cells and identification of heparin S^{35} in the mast cells was accomplished as follows: The sucrose-mast cell suspension from each rat was treated by dilution with 1 ml of a freshly prepared mixture of sodium chloride (.85%) and gelatine (0.01%) in versene phosphate buffer pH 7.4(7). The mixture was centri-

fuged at 1200 rpm for 10 minutes, the supernatant was decanted and the "button" of concentrated mast cells was spotted on one end of a filter paper strip (Whatman No. 1 — ¾ × 7 inches). The strips were arranged for ascending chromatography using 4 parts 25% ethyl alcohol with 1 part 0.1 N NaOH as the mobile phase. The chromatograms were either sprayed with 0.2% toluidine blue for evidence of metachromatic activity or the section of paper at the level of the heparin band was eluted with physiological saline and tested for anticoagulant activity and isotope activity.

Results. Distribution of heparin S^{35} in rat tissues at 2, 6, 12, 18 and 24 hours post injection is shown in Fig. 1. Initially the plasma contained approximately 20 times the radioactivity of the cells and plasma isotope activity decreased to nearly zero in the first 12 hours. Activity in the liver and spleen decreased more slowly so that minimal radioactivity continued to be present for 18 to 24 hours.

Metachromatic material having anticoagulant action *in vitro* which was abolished by protamine was obtained by paper chromatography from the peritoneal mast cells of a single rat. The eluate from the paper showed 25 to 60 cpm. Radioautographs of smears of these cells showed isotope activity in the granular material.

Plasma S^{35} anticoagulant activity and urinary excretion of S^{35} following injected heparin S^{35} (3.0 mg/kg) was determined in 3 dogs. Similar results were obtained in each animal and Fig. 2 shows results obtained in 1 experiment. The figure shows that anticoagulant effect parallels the radioactivity of the sample. Excretion of the isotope in the urine is synchronous with the decrease in blood concentration and anticoagulant activity. This indicates that the short duration of systemic anticoagulant effect is due in part to elimination of the drug from the circulating blood by the kidney. However, only 15 to 20% of injected S^{35} was found in the pooled urine at the time when coagulation time of the blood had returned to normal.

Discussion. Eiber and Danishefsky(4) es-

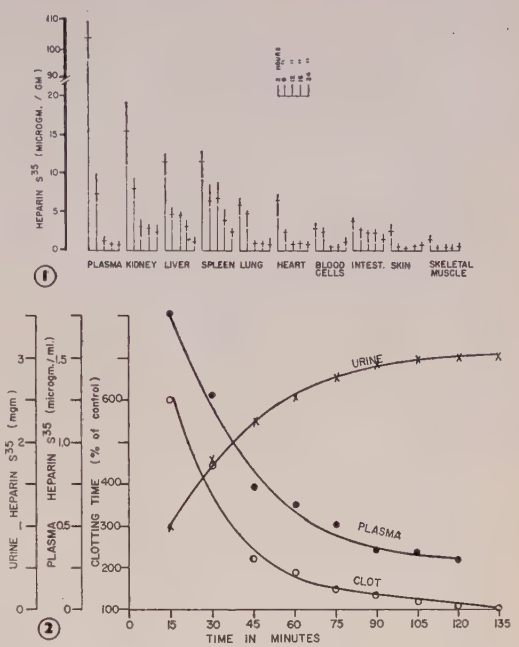

FIG. 1. Distribution of intrav. administered heparin-S^{35} (20 mg/kg) in rats at 2, 6, 12, 18 and 24 hr post-inj. Each column indicates avg and range of results obtained in 4 rats. Weight of heparin-S^{35} indicated on ordinate calculated on basis of 6,300 cpm/mg.

FIG. 2. Whole blood clotting time, plasma isotope concentration and excretion of isotope in urine at 15 min. intervals following intrav. inj. of heparin-S^{35} (3 mg/kg) in a 6 kg dog. Weights of heparin-S^{35} indicated on ordinates calculated on basis of 6,300 cpm/mg. Excretion of isotope in urine is represented on a cumulative basis.

tablished that when labeled inorganic sulfate is injected into dogs, it is incorporated into liver heparin. They concluded that heparin is synthesized in the intact animal from glucose and inorganic sulfate and showed the product to have a half life of about 3½ days. These workers have also indicated that circulating heparin may exist in part as a complex and that total anticoagulant activity can be determined only after proteolytic digestion(3).

The current study indicates that heparin S^{35} does not appear in significant amounts in the blood cells and that moderate doses of heparin are rapidly cleared from the plasma. Since intensity and duration of anticoagulant effect parallel isotope concentration in plasma, administered heparin appears not to be bound or complexed in inactive form in the

circulating blood. Urinary excretion accounts for only approximately 20% of injected heparin and therefore does not account for the rapid elimination of anticoagulant from the blood. Liver and spleen appear to retain isotope activity after anticoagulant and isotope activity have been eliminated from the plasma.

Mast cells are believed to be a site of synthesis or storage of heparin in the intact animal. Spolter and Marx(8) have shown that isolated mouse mastocytoma is capable of synthesizing heparin from inorganic sulfate. These workers have also shown that phosphoadenosine-phosphosulfate serves as the sulfate donor for synthesis of heparin by the mouse mastocytoma. The intense metachromatic activity of mast cell granules is believed to be due to presence of mucopolysaccharides having heparin-like activity. The current experiments indicating that injected heparin S^{35} appears in the granules of peritoneal mast cells of the rat suggest that the mast cells may function as a storage depot for administered heparin.

Summary. Heparin S^{35} was extracted from the liver of dogs administered inorganic $Na_2S^{35}O_4$. Distribution of this heparin was determined in rats. The mast cells were found to take up injected heparin S^{35}, and radioactivity persisted in liver and spleen after virtual disappearance from plasma. Anticoagulant activity was found to parallel isotope activity of the plasma of dogs injected with heparin S^{35}. Urinary excretion in dogs accounted for only about 20% of the injected isotope at a time when plasma anticoagulant activity had returned to normal.

Technical assistance of Miss C. S. Spaeth is gratefully acknowledged.

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Effect of Thyroxine on Enzymes of the Urea Cycle in Regenerating Rat Liver.* (26380)

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Studies in this laboratory have shown(1,2) that treatment of *R. catesbeiana* tadpoles with low concentrations of L-thyroxine (2.6×10^{-8}M) resulted in a marked increase in the specific activity of liver carbamyl phosphate synthetase. This increase has been shown to be due to *de novo* synthesis of the enzyme(3). An increase in specific activity has also been observed for 2 other urea cycle enzymes, namely ornithine transcarbamylase and arginase, during thyroxine-induced metamorphosis (unpublished studies).

In view of these findings, it seemed desirable to determine whether the thyroid hormone played a similar role in mammalian tissues. For this purpose, the effect of thyroidectomy on the activity of carbamyl phosphate synthetase, ornithine transcarbamylase and arginase in regenerating rat liver was investigated.

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TABLE I. Liver Weight during Regeneration in Athyroid Rats.

Animal No.	Preoperative body wt (g)	Liver removed (g)	Body wt before sacrifice (g)		Wt of liver after regeneration (g)			g liver/100 g body wt
			Athyroid	Athyroid plus thyroxin	48 hr	72 hr	144 hr	
14	173	3.35	163		2.75			1.69
15	193	3.50	190		2.55			1.34
22	160	3.45	160		2.60			1.62
12	168	2.55		153	3.30			2.16
20	175	3.00		160	3.40			2.13
21	152	3.15		142	2.70			1.90
1	160	2.55	160			2.30		1.44
2	155	3.10	152			2.25		1.48
3	175	3.70	172			3.10		1.80
5	150	2.70		126		4.10		3.25
6	165	2.95		149		4.75		3.19
10	175	3.55	178				4.90	2.75
19	175	3.80	185				4.40	2.38
8	165	3.55		125			3.35	2.68
9	152	2.85		128			8.30*	6.48
17	240†	5.20		230			8.10	3.52

* Histochemical examination showed fat infiltration.
† Body wt was 160 g when experiment was started.

Methods. Male albino rats,‡ weighing 145-180 g were used. One week after surgical thyroidectomy, each rat received an intraperitoneal injection of 500 μcuries carrier-free I^{131}. Following this, the animals were kept in metabolic cages and were fed a low iodine diet§ enriched in protein by the addition of 10% crude casein. A second dose of 250 μcuries I^{131} was administered 2 weeks after the first injection. Partial hepatectomy by the usual technic(4), with removal of 65 to 75% of the liver, was performed under light ether anesthesia 6 to 8 weeks after administration of the last dose of radioactive iodine.

Some of these animals (designated "athyroid plus thyroxine") received subcutaneous injections of 0.5 mg L-thyroxine during the period that elapsed between partial hepatectomy and sacrifice. Rats 4-9, and 12 were given an additional injection of L-thyroxine 12 hours before partial hepatectomy. The liver samples were carefully blotted with filter paper, weighed, and the enzymes assayed in duplicate.

Assay procedures and units of enzyme activity are those previously described(5). Protein determinations were carried out according to Lowry *et al.*(6) and liver glycogen was estimated by the anthrone method(7).

Results and discussion. The rate of liver regeneration is shown in Table I and Fig. 1. It is apparent from the table that the athyroid animals show a slower rate of liver regeneration and that injection of thyroxine improves the proliferative capacity to levels that compare with those of normal rats kept on the same diet (Fig. 1). Following partial hepatectomy average daily food intake dropped to about one-half and one-third that of the intact animals, respectively, for the athyroid and "athyroid plus thyroxine" rats. No significant difference could be found for total liver protein content on a wet weight basis among all 3 groups of rats. Glycogen estimations were used as an additional indication of degree of response to thyroxine administration(8). The glycogen content was below level of detection in all thyroxine-injected animals.

The influence of thyroidectomy on liver regeneration in the rat has been studied by Christensen and Jacobsen(9) and by Drabkin(10) who concluded that thyroidectomy did not impair liver regeneration. However, these investigations dealt with liver weight

‡ Supplied by Holtzman Co., Madison, Wisc.

§ The low iodine diet used and the casein used for enrichment were purchased from Nutritional Bio-Chemicals Corp., Cleveland, Ohio.

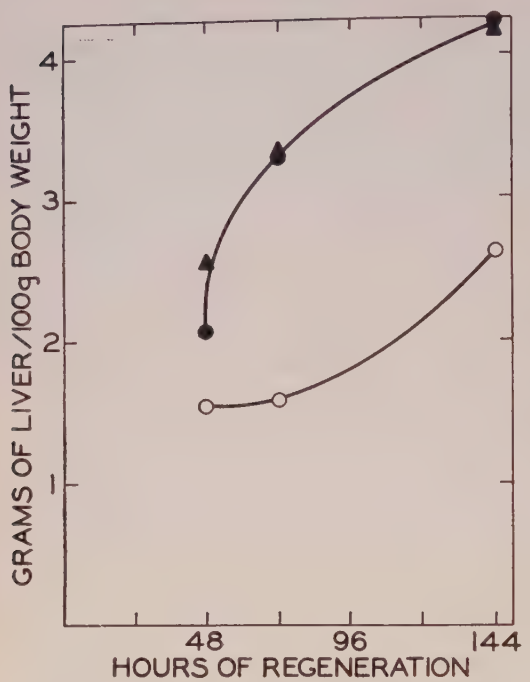

FIG. 1. Rate of liver regeneration in normal (▲—▲), athyroid (○—○) and athyroid plus thyroxine (●—●) rats.

estimations in the time period starting 2 weeks after partial hepatectomy and thus did not deal with the early stages of the regeneration process. Further, only surgical thyroidectomy was employed in these studies.

As to the activity of the enzymes measured,‖ no significant differences between normal, athyroid and "athyroid plus thyroxine," could be found in the livers of these 3 groups of rats at time of hepatectomy (excised liver), or during regeneration with the possible exception of ornithine transcarbamylase (Table II).

Values for activity of ornithine transcarbamylase in excised liver did not show significant differences among the 3 groups of rats (Table II) However, athyroid animals failed to reproduce the consistent fall in specific activity that is known to occur during regeneration in the livers of normal rats(11). Furthermore, injection of thyroxine to these animals resulted in a steady decrease in specific activity of ornithine transcarbamylase. The hypothesis has been previously proposed (11) that the decrease in specific activity of ornithine transcarbamylase that takes place during liver regeneration, together with a simultaneous increase in specific activity of carbamyl phosphate-aspartate transcarbamylase(12) might represent a regulatory mechanism by which more carbamyl phosphate is made available for pyrimidine biosynthesis.

The present results, considered in relation to the regeneration process, seem to lend further support to this proposal.

An additional point deserves some comment. While in *R. catesbeiana* tadpoles, thyroxine apparently "unlocks" a mechanism leading to an increased rate of synthesis of carbamyl phosphate synthetase, ornithine transcarbamylase and arginase, the thyroid hormone is not required for production of these urea cycle enzymes by newly formed rat liver cells. The different response of the amphibian and mammalian species is better appreciated when it is realized that thyroxine injection to athyroid, partially hepatectomized rats, resulted in a non-specific stimulation of liver protein synthesis.

Summary. The activities of carbamyl phosphate synthetase, ornithine transcarbamylase and arginase have been found to be unchanged in regenerating liver of thyroidectomized rats. Complete thyroidectomy results in an impaired rate of liver regeneration. Daily injection of thyroxine during the

TABLE II. Ornithine Transcarbamylase Activity in Liver of Athyroid Rats.*

Time after hepatectomy (hr)	Athyroid (specific activ.)	Athyroid plus thyroxin (specific activ.)
0	323 ± 62 (13)	331 ± 58 (8)
48	301 ± 7 (3)	244 ± 55 (3)
72	338 ± 43 (3)	232 ± 25 (2)
144	258 ± 30 (2)	186 ± 17 (3)

* Values represent avg specific activity ± stand. dev. Figures in parentheses indicate No. of animals.

‖ Other enzymes measured in some of these animals include glutamic dehydrogenase and carbamyl phosphate-aspartate transcarbamylase. Values obtained for specific activity of these enzymes were within the expected range. We are grateful to Dr. N. de Groot for glutamic dehydrogenase assays, and to Dr. S. Kim for aspartate transcarbamylase assays.

regeneration period restores the capacity of the liver to regenerate.

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Modification of Lethality of Endotoxin in Mice by Zymosan. (26381)

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Benacerraf *et al.*(1) have shown that pre-treatment of mice with the yeast extract, zymosan, increases susceptibility to lethality of *intravenously* administered endotoxin, despite the concomitant stimulation of phagocytic activity of the reticuloendothelial system commonly associated with refractoriness to endotoxic effects. We decided to determine whether plasma of such RES-stimulated but endotoxin-susceptible mice would protect normal recipients against lethality of endotoxin, as does plasma of RES-stimulated endotoxin-tolerant donors(2). Mice were treated with an available zymosan preparation, plasma taken from some, the others being challenged *intraperitoneally* with endotoxin. Surprisingly, these zymosan-treated donors showed increased resistance to the *i.p.* endotoxin challenge.

The present report confirms the finding of Benacerraf *et al.*(1), for the experimental model they used, and describes the modification of endotoxin susceptibility by zymosan pre-treatment, ranging from increased resistance to increased susceptibility, depending upon the zymosan employed, method of preparation for injection, and route of administration of the endotoxin challenge. Stimulation of phagocytic activity was observed uniformly and the protection seen was not attributable to contamination by bacterial endotoxin or to stimulation of antibody formation to the challenging endotoxin.

Materials and methods. For studies on modification of endotoxin lethality and phagocytic activity, mice of both sexes, weighing 16-20 g, were used. Experiments to determine possible endotoxin contamination of zymosan were performed on male rabbits weighing 2 to 2.5 kg.

The results obtained with 2 zymosan preparations, designated "A" and "B",* are reported herein. The suspension of zymosan was prepared either exactly as described by Benacerraf *et al.*(1) or by substituting vigorous boiling directly on a hot plate for the lesser degree of heating in a boiling water-bath. Precautions to avoid endotoxin contamination were observed: all vials, beads, syringes, and needles were heated in an oven at 175°-180°C for 2-3 hours before use. Saline for dilutions was demonstrated to be pyrogen-free by test in rabbits. Pre-treatment of mice was standardized at 1 mg in 0.2 ml,

* Zymosan "A" was obtained from Mann Res. Labs., lot #4393, and "B" from Fleischmann Labs., lot #9B551.

intravenously, on days 1 and 3, with testing for endotoxin susceptibility and phagocytic activity, in separate groups of mice, on day 4. The endotoxin used was the lipopolysaccharide of *S. typhosa* 0 901 (Difco), administered either *i.p.* or *i.v.* at varied doses as indicated below. Deaths were recorded for 72 hours, with most occurring within 24 hours.

Clearance of carbon from the blood for determination of phagocytic activity of the RES was measured by the method of Biozzi *et al.*(3), as modified in our laboratory. We have previously shown that daily doses of more than 1 μg of endotoxin are required to protect against an LD_{75} challenge (0.6 mg) of the lipopolysaccharide used here(2). It is clear, then, that the 1 mg daily mouse-dose of zymosan would have to have an endotoxin contamination of not less than 0.1% for this to explain the increased resistance to the challenge. The normal rabbit responds to *i.v.* endotoxin, in μg doses, with a characteristic biphasic fever and an acute leucopenia. To test for contamination, zymosan preparations were injected *i.v.* at a 10 mg dose following which rectal temperatures and peripheral white cell counts were recorded. The preparation of rabbits for, and the handling during, the testing have been described(4). The sensitivity of the test is such that less than 0.01% contamination (<0.1 μg/mouse-dose zymosan) would be revealed.

Zymosan has been shown to be antigenic, capable of increasing bactericidal antibody in rabbits, and also to enhance hemolysin production in the rat(5,6). Therefore, the possibility existed that the zymosan administration used in these experiments stimulated production of antibody reactive to the challenging bacterial endotoxin employed. Thus sera pooled from 6 normal mice and from the same number of mice treated with representative zymosan preparations were collected on the 4th day of treatment and kept at −20°C until used. The pooled sera were then titrated for antibody to *S. typhosa* 0 901 lipopolysaccharide based on the method of Neter *et al.*(7), using sensitized sheep red blood cells.

TABLE I. Modification of Endotoxin Lethality by Zymosan "A," Prepared on Hot Plate.

Group	Route of challenge	No. of exp.	Dead/ Total	% dead
Controls	i.p.*	3	21/30	70
Zymosan-treated	"	3	9/30	30
Controls	i.v.†	3	11/30	37
Zymosan-treated	"	3	11/30	37

* .4 to .6 mg endotoxin in individual exp.
† .1 to .2 " *idem*

Results. Susceptibility to endotoxin challenge. In Table I are summarized the data demonstrating that pre-treatment with zymosan "A", prepared for injection by boiling directly on a hot plate, resulted in increased resistance to intraperitoneal endotoxin challenge, without alteration of normal susceptibility to intravenous challenge. Treatment with zymosan "A" prepared in the water-bath resulted in increased susceptibility to *i.v.* challenge (controls 2/10 dead, zymosan 6/10).

With the hot plate treatment, zymosan "B" (Table II) did not alter the susceptibility to *i.p.* challenge and increased susceptibility to *i.v.* challenge. When this zymosan was prepared in the water-bath, increased susceptibility to both routes of challenge was found, with much greater sensitivity to the *i.v.* route. These results confirm those of Benacerraf *et al.*(1).

In an attempt to determine whether the increased susceptibility to endotoxin seen 24 hours after the last dose of zymosan "B" was a transitory effect, mice were given 1 mg of the water-bath suspension *i.v.* on days 1, 3, and 6, and groups of 10 mice each were challenged with 0.6 mg endotoxin *i.p.* on

TABLE II. Modification of Endotoxin Lethality by Zymosan "B" Pre-treatment; Boiling Water-Bath *vs* Hot Plate Preparations.

Group	Route of challenge	No. of exp.	Dead/ Total	% dead
Controls	i.p.*	4	15/40	38
Zymosan, water-bath	"	3	20/30	67
" , hot plate	"	3	13/30	43
Controls	i.v.†	4	5/40	13
Zymosan, water-bath	"	5	22/50	44
" , hot plate	"	3	8/30	27

* .4 mg endotoxin. † .1 mg endotoxin.

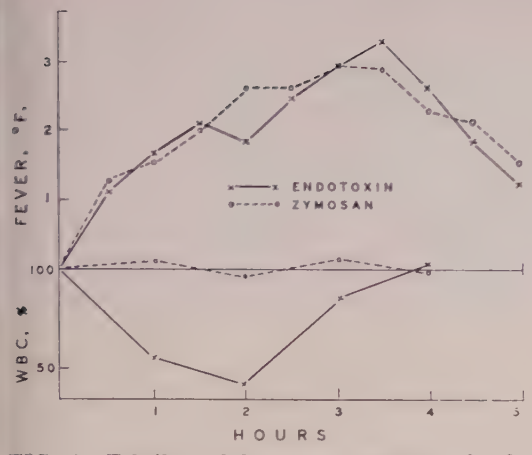

FIG. 1. Febrile and leucocyte responses of rabbits given 10 μg *S. typhosa* endotoxin or 10 mg zymosan "A," i.v.

days 7, 8, 9, and 10. No evidence for development of a resistant state was obtained.

Stimulation of RES. Both zymosan preparations produced equivalent stimulation of phagocytic activity as measured by rate of clearance of carbon from the blood 24 hours after the second injection, the time of testing for endotoxin susceptibility. At a dose of 12 mg/100 g body weight, the half-times for carbon clearance were: controls, 11 minutes; zymosan "A", 6 minutes; zymosan "B", 6.5 minutes.

Test for endotoxin contamination. The distinction between endotoxin and zymosan "A" as regards course of fever induced and peripheral leucocyte response is described in Fig. 1. Both "A" and "B" zymosans were equally pyrogenic, producing monophasic fevers differing qualitatively from the typical biphasic endotoxin fever shown. The minimum endotoxin contamination to account for the protective effect of zymosan "A" in the mouse would require that the 10 mg dose given these 7 rabbits contain 10 μg, the dose used in the 5 endotoxin-treated rabbits. It may be seen that the total magnitude of fever (area under the curve) is the same for both substances, lending emphasis to the qualitative difference in pattern at this level of fever. The acute leucopenia characteristic of endotoxin(8) was not elicited by either zymosan.

Antibody titer to S. typhosa endotoxin. The sera taken from normal mice demonstrated no detectable antibodies to *S. typhosa* 0 901 lipopolysaccharide as measured by the sensitive hemolysis technic. The sera from mice pre-treated with zymosan "A" or "B" likewise gave no evidence for the existence of any detectable antibodies to the challenging endotoxin.

Discussion. Zymosan is a yeast cell wall fraction, principally polysaccharide, but known to contain some lipid and protein. There is no primary chemical standard, standardization being based upon immunological properties(9). With the demonstration that one zymosan preparation may protect against endotoxin lethality while another, producing equally great stimulation of phagocytic activity, increases susceptibility, one is led to look for a "contaminant," defined in terms of other than RES-stimulating activity. Since available zymosan preparations are not sterile, the possibility of endotoxin contamination immediately suggests itself, and such contamination could explain increased resistance. This has been shown not to be the case: no evidence for endotoxin contamination was found. The pyrogenicity of zymosan, equally present in protective and sensitizing zymosans, is similar to that of other polysaccharides and distinctly different from that of bacterial endotoxin at doses producing equivalent fevers. The absence of an acute leucopenic response provides a further distinction from endotoxic activity.

On the supposition that an anamnestic reaction might occur in our mice which might have normal antibody cross-reactive with the challenging endotoxin, or more remotely that *de novo* antibody might appear stimulated by zymosan treatment, the hemolysis tests were conducted. However, since no detectable circulating antibody was found either in normal or treated mouse sera under our experimental conditions, it is clear that such an immunological mechanism is not operable here.

It is clear that preparations of zymosan equivalent in one activity may be quite different in another. Zymosan administered *i.v.* is not without toxicity and it would seem

reasonable to consider a heat labile toxic contaminant variably present which may mask the protective effect afforded by RES stimulation. Benacerraf's findings(1) suggest a deleterious effect upon adrenal cortical function, which doubtless plays a permissive role in resistance to endotoxin. The relatively greater sensitivity of zymosan-treated mice to *i.v.* than to *i.p.* endotoxin challenge, compared to normal controls challenged by the same routes, was found regardless of the type or preparation of zymosan. The slower absorption of the *i.p.* endotoxin challenge may allow a more effective detoxification by the RES in a pre-existing state of susceptibility.

Passive transfer experiments require further study; protection was afforded normal recipients by plasma of resistant zymosan "A"-treated donors, but, peculiarly, plasma of donors given zymosan "B" conferred neither protection nor the donors' own susceptibility to endotoxin challenge.

Summary. Pre-treatment of mice with different preparations of zymosan modified the lethal effect of a subsequent endotoxin challenge, ranging from increased resistance to increased susceptibility, despite equivalent RES stimulation. Source and degree of heating in preparation for injection were determinants. Greater relative sensitivity to *i.v.* than to *i.p.* challenge was found regardless of the zymosan used. Protection was not attributable to endotoxin contamination or to stimulation of serum antibody titer to the challenge. Zymosan produced a monophasic fever in the rabbit, differing from the biphasic endotoxin fever, and did not elicit an acute leucopenia.

The skilled technical assistance of Miriam Graff is gratefully acknowledged.

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Apparatus for Simultaneous Infusion and Multiple Blood Sampling. (26382)

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Continuous withdrawal of simultaneous arterial and venous blood samples is required in experimental procedures such as determination of regional blood flow(1,2). This can be done with some commercially available pumps. Rose and Pfaff(3) have described a mechanism designed specifically for this purpose. The design of these pumps, however, requires syringes to be placed at a distance from the experimental animal. This necessitates a long cannula with consequently a considerable volume of "dead space." Also, simultaneous infusion and withdrawal are possible with available commercial pumps only by using 2 separate units.

A description follows of an apparatus designed specifically for use in regional blood flow studies. Two objectives were kept in mind, (a) to place the syringes as close as possible to the experimental animal and thus reduce cannula length to a minimum, (b) to

* Contribution No. 9, Engineering Research Service.

† Contribution No. 48, Animal Research Inst.

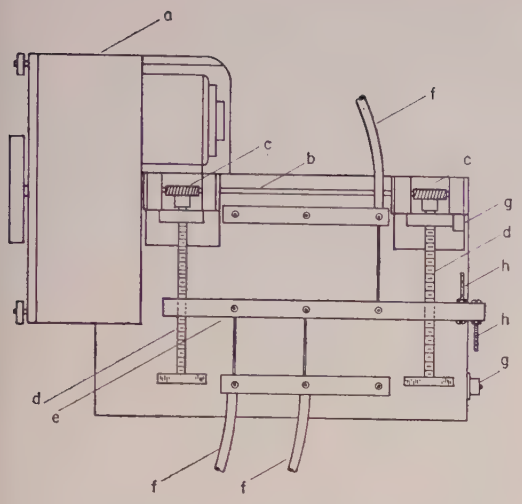

FIG. 1. Variable-speed drive assembly. (a) Harvard multi-speed transmission, (b) drive shaft, (c) worm gears, (d) lead screws, (e) drive bar, (f) push-pull wire choke assembly, (g) sub-micro limit switches, (h) contact screws.

allow simultaneous infusion and withdrawal.

Description of unit. The infusion-withdrawal pump is composed of 2 main components, a variable speed drive assembly and remote-operated syringe units. The drive assembly (Fig. 1) is powered by a Harvard Multi-Speed Transmission.‡ The output shaft of the Harvard unit (a) is connected by a flexible coupling to a drive shaft (b) having 2 worm gear drives (c) spaced approximately 7 inches apart. The worm gears drive 2 lead screws (d) which impart linear motion to a drive bar (e). The speed reduction of the worm gears is 5:1 and the lead screws are cut 16 threads per inch. A flexible push-pull wire choke assembly (f), approximately 18 inches long, transmits the linear motion of the drive bar to each remote-operated syringe unit. Travel of the bar is limited by sub-micro limit switches (g)

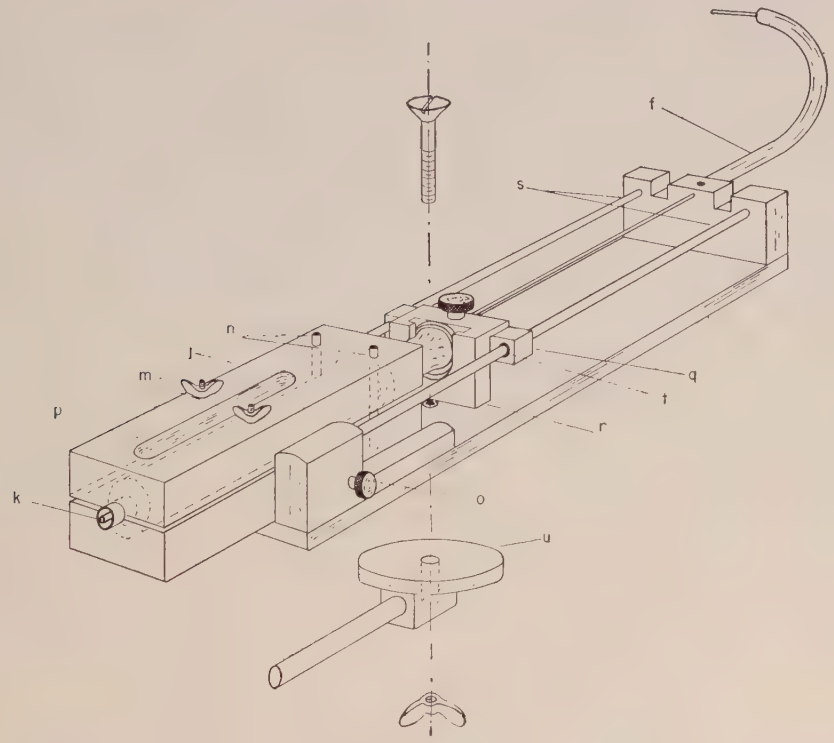

FIG. 2. Syringe unit. (j) Plexiglass split-block, (k) Luer-Lok connector, (m) bolts and wing nuts, (n) locating pins, (o) thumb screws, (p) slot in block, (q) sliding block, (r) interchangeable holder, (s) guide rods, (t) teflon bushings, (u) swivel base.

‡ Harvard Apparatus Co., Inc., Dover, Mass., Cat. No. 600-000.

in series with the Harvard unit. The switches are activated by contact with screws (h) threaded into the drive bar. Direction of travel of the drive bar can be reversed by means of a 3-position toggle switch on the reversible synchronous motor.

In the syringe unit (Fig. 2), the barrel of the syringe is held in an interchangeable plexiglass split-block (j) bored to size and fitted so the Luer-Lok connector (k) protrudes beyond the end of the block. Compression of the split-block by 2 bolts and wing nuts (m) holds the syringe barrel securely in position. The block in turn is positioned on the base of the unit by 2 locating pins (n) and held by 2 thumb screws (o). The top portion of the block is slotted (p) to give unobstructed view of the graduations on the syringe.

The flanged end of the syringe plunger is attached to a sliding block (q) by an interchangeable holder (r). The sliding block, which operates on 2 guide rods (s), is actuated by the push-pull wire choke assembly (f). Teflon bushings (t) in the sliding block reduce friction on the guide rods to a minimum. Each syringe unit is equipped with a swivel type base (u) for greater flexibility in positioning.

At maximum gear speed the linear speed of the syringe plunger is 0.75 in/min. This can be reduced in 12 steps to 1/5000 of the maximum rate. The movement of the plunger may become jerky at very low speeds but this can be overcome by using a syringe with a loosely fitting oiled plunger.

Simultaneous infusion and withdrawal can be achieved by attaching a wire choke assembly on either side of the drive bar. While simultaneous infusion and withdrawal must be carried out at the same gear speed, variation is possible in the choice of syringe size and concentration of infusate.

Discussion. Volume and variability of delivery were calculated from the weight of mercury delivered during a known time interval. Maximum rates of delivery with 2.0, 5.0 and 10.0 ml syringes were 1.181 $\pm$ 0.008, 2.034 $\pm$ 0.006 and 3.214 $\pm$ 0.006 ml per min, respectively (mean of 5 to 15 trials per syringe $\pm$ standard deviation). Variability of delivery at other speeds was comparable to that given for maximum rates.

Due to a certain degree of play in the cables, there is a time lag between switching the instrument on and movement of the syringe plunger. This lag is only a fraction of a second at higher speed settings but increases proportionally as rate of delivery or withdrawal decreases. This, however, is a practical problem only when operating at very low rates for a short period of time.

Movement of the cables after the apparatus has been switched on will result in a slight displacement of the syringe plunger. Therefore, care must be exercised not to move the cables accidentally after an experiment has started.

Summary. An infusion-withdrawal apparatus is described which eliminates the necessity for long cannulae between syringes and experimental animal and permits multiple infusion and withdrawal to be carried out simultaneously. Engineering plans are available from the senior author.

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Effect of Hypoxia on Plasma Erythropoietin in the Rabbit.* (26383)

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It has been shown previously in the rat(1) that plasma erythropoietin (EPF) rises significantly in a short period (4-8 hours) in response to a hypoxic stimulus. This rise in EPF reaches a maximum at 24 hours and returns to normal at 48 hours even though the animal remains in a hypoxic atmosphere. Others have demonstrated the same general time response pattern(2). The present study was carried out to determine whether rabbits would respond similarly to a hypoxic stimulus.

Materials and methods. New Zealand white rabbits were kept at 10 vol % O_2 concentration by methods previously described (1). At various intervals, rabbits were removed from the chamber and immediately exsanguinated by bleeding *via* heart puncture. The heparinized plasma was prepared for assay by 2 methods; the heat deproteinization procedure of Borsook, *et al.*(3) and step 1 procedure of White, *et al.*(4). In both methods, the final material was concentrated 3 times when compared to the original volume of plasma. The plasma extracts were assayed in starved rats using the method of Fried *et al.*(5) and Mirand *et al.*(6).

Results. The results are summarized in Table I. Significant elevation of plasma EPF levels was evident at 8 hours. Peak levels were present at 24 and 48 hours. By 72 hours the level of EPF had returned to normal levels and remained there up to 120 hours. The "step 1" plasma extract was slightly more active than the heat deproteinized extract.

Discussion. These results demonstrate that the rabbit and the rat respond similarly to a hypoxic stimulus. The only demonstrable difference is that erythropoietin levels may remain elevated slightly longer in the rabbit than the rat(7).

Current interest in the chemistry of erythropoietin makes a quick, simple source of active material desirable. The above data point to plasma of the 24 hour hypoxic rabbit as such a source. Of real importance also is the question whether hypoxic EPF differs from post-hemorrhagic or anemic EPF. Previous studies in our laboratory(7,8,9) and elsewhere(10) have shown that EPF can be elicited in the plasma of hypoxic, nephrectomized rats. However, no assayable quantities of EPF appear in bled, nephrectomized rats(8,11,12). This suggests different sources of hypoxic and post-hemorrhagic

TABLE I. Erythropoietin Titer, Determined by 24 Hour Fe^{59} Uptake, of Starved Rats That Received Plasma from Rabbits Exposed to 10% O_2 for 0 to 120 Hours.

Plasma from hypoxic rabbits at hour	No. of rabbits	White step 1 plasma extract		Modified Borsook plasma extract	
		24 hr Fe^{59} uptake, %	Hematocrit, %	24 hr Fe^{59} uptake, %	Hematocrit, %
0	8	5.4 ± 1.1*(12)†	51.9	5.2 ± .6 (5)	51.3
4	8	5.4 ± 1.9 (12)	48.4	3.4 ± .9 (5)	48.2
8	8	13.5 ± 2.0 (12)	51.6	5.7 ± 1.2 (5)	50.2
24	8	19.4 ± 3.1 (11)	53.9	13.2 ± 1.6 (5)	50.8
48	8	18.2 ± 3.2 (12)	53.0	12.9 ± 2.3 (5)	51.4
72	4	4.6 ± 1.5 (5)	46.8	4.6 ± 1.5 (5)	47.3
96	4	4.7 ± .9 (5)	51.2	4.6 ± 1.7 (5)	51.0
120	4	8.6 ± 1.8 (5)	51.4	4.6 ± 1.8 (5)	53.0

Data represents the avg of several assays, all of which showed the same pattern of response.
* Stand. dev. † No. of normal starved rats used in assay for erythropoietin.

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EPF. Do they also differ chemically?

These data also emphasize another problem: namely the differing time response of hypoxic EPF and anemic EPF. The former shows a quick rise and a quick reversion to normal. The latter goes up quickly after a significant anemia is established and remains up for long periods of time. We have recently assayed plasma from severely iron-deficient rats that have been anemic for 3 months(13). High values of EPF were found without exception. Does this differing time response likewise imply a different source and/or a different identity of hypoxic and anemic EPF? Detailed studies of the chemical properties of EPF produced by these various stimuli are in progress in our laboratory.

Summary. Rabbits have been placed in a low O_2 atmosphere for varying periods of time and plasma erythropoietin content determined after set intervals of hypoxia. EPF was found to rise significantly at 8 hours, reach peak levels at 24 and 48 hours, and return to normal at 72 hours.

The authors express their thanks for assistance of André Bulba, Ruth Deckert, Edward Dywinski, Helen Fox and William Sywenkyj.

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A Microbioassay for Acetylcholine.* (26384)

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Many classical researches reported in literature are concerned with the action of acetylcholine (ach). In such work there has often been a need to determine low concentrations of ach in small volumes of fluid or perfusate. Direct chemical analyses by methods such as the one described by Hestrin(1) are ordinarily too insensitive for these applications and one must turn to a bioassay. Minz(2) has summarized the most frequently employed bioassay procedures and has pointed out the advantages and disadvantages of each. There is, for example, an approach in which one measures the contraction of the dorsal muscle of the leech, a preparation which will respond to ach at a concentration of 5×10^{-8} M. This preparation is suspended in a bath whose volume is usually about 10 ml and therefore if a 0.1 ml sample of solution is added to the bath, this sample, if it is to be successfully assayed, must contain ach at a minimal concentration of 5×10^{-6} M.

We wished to conduct certain experiments

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wherein one must be able to determine low concentrations of ach in very small volumes of fluid. To achieve this goal we have combined several technics previously employed in this laboratory and thereby have devised a reliable method by which one may detect ach in concentrations as low as 2.5×10^{-8} M in samples whose minimum volume approaches 0.15 μl.

In this assay we have taken advantage of the well-known fact that the chronically denervated frog sartorius muscle becomes very sensitive to diffusely applied ach(3,4). In our method, the denervated endplate area of a muscle fiber is observed under a microscope while this site is microperfused with a solution whose ach concentration is to be determined. The microperfusion is accomplished using a glass micropipette of tip diameter 20-40 μ. Such pipettes are manufactured from 1.5 mm O. D. Pyrex tubing using only the weak pull of the micropipette puller designed in this laboratory(5). A small volume of the sample solution is drawn into the pipette whose shank is then thrust into a short length of plastic tubing, the other end of which is slipped over the tapered end of a glass tube. This assembly is mounted at a 45° angle in a micromanipulator. To the upper end of the glass tube is connected another length of plastic tubing to which is applied a pulse of air pressure sufficient to eject the sample. For this purpose it is convenient to use a pressure bottle, an air filled hypodermic syringe or one may apply pressure by mouth.

At the start it is best to become familiar with the technic by using normal frog sartorius muscle. The dissected normal muscle is mounted flat in a chamber filled with Ringer's solution and this assembly is placed on a microscope stage in order that the transilluminated muscle can be observed at 100-150 $\times$ magnification(6,7). Thereafter the Ringer's bath solution is drawn off and replaced at 20 min intervals. It is essential to use an optical system in which the objective and condenser have an N.A. of at least 0.25 in order to obtain the necessary resolving power (cross striations should be clearly visible). On scanning the medial surface of the muscle one may easily locate main nerve branches and these are followed to their various terminations where groups of endplates are located. The tip of the loaded perfusion pipette is placed immediately above but not touching a single endplate. If the threshold concentration of ach is present in the sample, then shortly after the perfusion begins, a brief easily observed tetanus will be evoked in the perfused muscle fiber. At higher concentrations of ach, the contractile response in this fiber will be briefly sustained after the perfusion is terminated and, adjacent muscle fibers, whose endplates lie nearby, will also respond. At the lower concentration of ach at which a reproducible contractile response may be obtained (ach threshold), the latency of the response averages about 1 sec and rarely exceeds 5 sec. We have found the ach threshold to lie in the concentration range $1.1\text{-}2.2 \times 10^{-5}$ M. At an endplate whose ach threshold is 2.2×10^{-5} M one cannot obtain the contractile response when ach at 2.09×10^{-5} M is applied. If determinations at a single endplate are repeated at 10 to 15 min intervals in order to permit recovery from receptor desensitization due to applied ach(8), then the ach threshold can be shown to remain constant over at least a 5 hr period. We have not attempted an endurance test for normal muscle but it is likely that such preparations when properly treated, remain useable for even longer periods (see below).

When a chronically denervated sartorius muscle is used, the assay sensitivity increases approximately 1000 fold. The procedure in this case is as follows. Under urethane anesthesia the hind leg of a frog is denervated by severing spinal nerves 7-9. The nerves are approached from the dorsal body surface, about 1 cm of nerve is removed, and the central stump is sutured to the skin thereby hindering reinnervation. Such denervated frogs are maintained for 2 months or more in a tank with running tap water at 15°C. Survival time is increased and the size of the muscle fibers is better maintained by force-feeding the frogs every second or third day with small pieces of liver(9).

At an appropriate time, the chronically denervated muscle is dissected and mounted as described previously. Identification of the endplate areas in this preparation is more difficult but with some experience and a good optical system one can locate these sites by following the old nerve pathways which remain marked by the surviving connective tissue elements. If this guide is unsuccesful, a trial and error testing with ach perfusion will reveal sites of greatest sensitivity. After 2 months denervation, the average ach threshold at endplate sites is 2.8×10^{-8} M (range $0.55\text{-}16.5 \times 10^{-8}$ M). Rate of development of this chemosensitization depends on the temperature at which the frogs are kept. One may take advantage of the range in sensitivity of a group of endplates to obtain a quantitative estimate of the concentration of ach in an unknown solution by applying it to several sites of different ach sensitivity. It may, of course, be necessary to dilute the unknown solution to place its final ach concentration within the range of the ach thresholds.

The ach threshold for each endplate site remains stable and reproducible for many hours. It is likely that the same muscle can be used for even longer periods because we have been able to obtain stable ach thresholds for muscles tested daily over periods of 2 to 4 days. These muscles were stored at 4°C during idle periods and at these times the Ringer bath was unchanged.

In trying to determine the maximum sensitivity of this method, we were able, using chronically denervated muscle, to obtain a contractile response when an ach containing solution was ejected from a typical micropipette (I. D. 27 μ) at the rate of 0.15 μl/sec. The amount of ach delivered, in the one second period required to produce a response, amounted to approximately 10^{-15} moles. One can see therefore that this method of delivering ach to the receptor site is relatively wasteful since by the iontophoretic method a single twitch can be evoked by applying only 10^{-15} to 10^{-16} moles of ach to a *normal* endplate(10,11). It would therefore appear that our assay could be increased in sensitivity by further refinements, but perhaps we are limited by having already approached the minimal *concentration* of ach which must be applied in order to produce a contractile response.

Some further increase in assay sensitivity could have been obtained by using an internal microelectrode to measure, as an index, a subthreshold reduction in transmembrane potential produced during the ach perfusion. This advantage may be outweighed because the technic is more laborious, technically demanding, requires more equipment, necessitates determination of a dose response curve for each area tested, and since the membrane must be punctured, there is a limit on the repeated use of each endplate. We may also add that addition of physostigmine sulfate (1.55×10^{-5} M) to the Ringer's solution bathing the chronically denervated muscle increases its sensitivity to ach only by a factor of 1.5 (avg). This is not surprising since there is evidence that the concentration of cholinesterase at the site of the denervated endplate in amphibian sartorius muscle decreases markedly by the 30th day after motor nerve section(12).

In this assay, the production of a muscle twitch involves reduction of the transmembrane potential to the critical value required for initiation of a propagated action potential. A sample may yield a positive response because it contains ach but such a response may also be obtained because, for example, the sample contains a high concentration of potassium ions. A spurious result of this type can be distinguished by adding d-tubocurarine to the Ringer's solution bath and to the sample. d-Tubocurarine will prevent depolarization by ach but not by potassium ions. Another means of differentiating between ach and potassium ions is to make use of the fact that ach has a greatly reduced depolarizing power at non-endplate sites while potassium ions are equally effective at all regions of the muscle fiber. Therefore one should test the sample both at the endplate and at an endplate free site such as may be found in the proximal portion of the pelvic end of the sartorius muscle.

Axelsson and Thesleff(13) have reported that one to 2 weeks post denervation, mammalian muscle becomes uniformly sensitive over its entire length to ach applied iontophoretically. Such preparations seemed to offer opportunity to improve our assay since they would eliminate the prolonged period of denervation and the need to identify endplate areas. Therefore we extended the study of the assay procedure to the denervated tenuissimus muscle of the cat, testing it 22 days after nerve section. Using our method of ach application we were able to initiate a contractile response at any site on the denervated muscle fiber but the ach concentration required to do this was 0.55-1.1 $\times$ 10^{-5} M, (a value close to the ach threshold for the endplate of an innervated fiber). As the concentration of ach solution applied was reduced, the region of the denervated tenuissimus fiber from which a response could be initiated became progressively smaller. The regions of greatest sensitivity (presumably corresponding to the endplate areas) had an ach threshold about 100 times less than that of the least sensitive regions. The highest sensitivity that we observed in a muscle denervated 22 days corresponded to an ach threshold of 5.5 $\times$ 10^{-8} M.

Kuffler(14) reported earlier that some amphibian muscle fibers which he examined several months after denervation seemed to be sensitive everywhere to ach applied in the form of small droplets. Recently Miledi, using the frog sartorius muscle(15) has made a detailed study of the progressive enlargement of the ach sensitive area with time after nerve section. Work done in this laboratory(9) confirms the fact that after prolonged periods of denervation the frog sartorius muscle becomes sensitive everywhere to ach, and as also reported by Kuffler and by Miledi, that a differential sensitivity between endplate and more remote areas persists for at least several months.

Summary. A microbioassay for acetylcholine is described in which a solution containing acetylcholine is microperfused onto the endplate area of a chronically denervated skeletal muscle fiber. The endpoint, a brief tetanus, can be produced by application of as little as 0.15 μl of a solution which contains acetylcholine chloride at a concentration of 2.5 $\times$ 10^{-8} moles/l.

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Electrophoretic and Immunologic Studies on the Chicken Serum and Egg Yolk. (26385)

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The serum of laying hens can be distinguished from that of cocks immunologically (1,2,3), chemically(4,5) and electrophoretically(6). The similarity(2,5,7) and difference(8) were noted between the phosphoprotein in the serum of laying hens and that in egg yolk. Hosoda *et al.*(9) using immunologic technics determined the content of the specific substance in the serum of laying hens and found that its titer correlates well with the ovarian activity of hens. Recently, Urist *et al.*(10) did chemical and electrophoretic studies on the serum of estrogenized cocks with aid of the ultracentrifuge.

The present work was initiated to elucidate the chemical nature of the antigens in specific immunologic reaction to the serum of laying hens or egg yolk, and the relationship between the specific substance in the serum of laying hens and that in egg yolk.

Materials and methods. Fifteen White Leghorn laying hens and cocks were sacrificed, and the serum and yolk in the follicles obtained from them were centrifuged in a Hitachi ultracentrifuge, at 40,000 rpm (105,400 $\times$ G) at 5°C for 8 hrs. Three partitions of the serum of laying hens, top layer (LSI) middle partition (LSII) bottom sediment (LSIII), 3 partitions of the serum of cocks, top layer (CSI middle partition (CSII) bottom sediment (CSIII), and 2 partitions of yolk, supernatant solution (YI) sediment (YII), were separated visually by ultracentrifugation. Partition YII was washed once with isotonic saline and ultracentrifuged again for 2 hours. Fractionation of phosphorus was carried out by Schneider's method(11). Phosphorus content of each fraction was determined by the method of Gomori(12). Nitrogen content was determined by the Kjeldahl method. Antisera was prepared by immunizing rabbits with the serum of laying hens, or yolk or the 5 ultracentrifuged partitions obtained from the serum of laying hens. Cock serum was added to these antisera to absorb species-specific antibodies. Sodium cyanide was added in a concentration of 0.1% to all the antigens and antisera for preservation. Interfacial precipitin reaction test was employed to measure antigenic potency, which was expressed as the highest dilution of antigen giving a positive reaction with each antiserum after 1 hr of incubation at 25°C. One cc of each antiserum previously absorbed with cock serum was mixed with 0.1-0.2 cc of a heterologous ultracentrifuged partition of the serum of laying hens or egg yolk. The antiserum-antigen mixtures were incubated at 37°C for 2 hr, and kept at 3-5°C overnight. The precipitates formed by the absorbing antigens were removed by centrifugation at 3,000 rpm for 10 min. Complete disappearance of the antigen was confirmed by the interfacial precipitin reaction test. Single diffusion precipitin test in agar-antiserum gel, according to the technic of Oudin(13) was used for the analysis of each partition. Test tubes of 4 mm diameter were used. Concentration of the antisera in the gel was 50%. 0.3 cc of the antigen diluted with the same amount of saline were placed upon the antiserum gel. The migration of precipitin zones was observed after 48 hr of incubation at 37°C. Filter paper electrophoresis was carried out by the method of McKinley *et al.*(14). Electropherograms of serum and yolk of the chicken and each ultracentrifuged partition of both the serum and yolk were made in barbital buffer, pH 8.6, containing 20% of methanol instead of water. Electropherograms were performed at 0.3 miliamperes per cm of filter paper for 7 hr, and stained with Amidoschwarz 10 B and Sudan III. Stained radioautographs were also made from the electropherograms of the serum and yolk, obtained from laying hens 24 hr after injection of 800 μc P^{32} as Na_2HPO_4, and of each ultracentrifuged par-

TABLE I. Distribution of Phosphorus, Nitrogen and Antigenic Potency among Partitions of Chicken Sera and Yolk Obtained by Ultracentrifugation.

Partitions	Acid soluble phosphorus	Lipid phosphorus	Protein phosphorus	Total nitrogen	Total protein	Antigenic potency*	Volume ratio
	μg/cc			mg/cc			
Laying hen serum							
Total (LST)	40	117	101	7.6	46.6	640	10
I (LSI)	0 (0)	530 (53)	0 (0)	3.1 (.3)	17.9 (1.8)	5,120	1
II (LSII)	36 (29)	67 (53)	5 (4)	4.1 (3.2)	25.1 (20.1)	80	8
III (LSIII)	116 (12)	200 (20)	899 (90)	46.5 (4.6)	290.6 (29.1)	5,120	1
Cock serum							
Total (CST)	60	64	0	4.1	24.8	0	10
I (CSI)	153 (23)	0 (0)	0 (0)	1.5 (.2)	8.4 (1.3)	0	1.5
II (CSII)	33 (41)	91 (75)	0 (0)	3.8 (3.1)	23.5 (18.4)	0	8.2
III (CSIII)	0 (0)	132 (4)	0 (0)	2.9 (.6)	18.4 (3.9)	0	.3
Yolk	μg/g			mg/g			
Total (YT)	250	5,000	1,045	27.7	164.9	20,480	10
I (YI)	244 (188)	5,247 (4,040)	52 (40)	21.9 (16.8)	129.0 (99.3)	10,240	7.7
II (YII)	261 (60)	1,957 (450)	4,126 (949)	40.5 (9.3)	253.3 (58.3)	40,960	2.3

* Expressed as the highest dilution giving a positive reaction with anti-laying hen serum absorbed with cock serum.

Figures in parentheses expressed in μg of partitions from 1 cc of serum or from 1 g of yolk.

tition of the serum and yolk. The corresponding fractions on unstained radioautograms were separated by cutting the paper, and extracted by dipping in saline overnight for detection of positive reaction on immunologic interfacial precipitin tests.

Results. Distribution of phosphorus, nitrogen, protein and antigenic potency (as determined by titrations with anti-laying hen serum absorbed with cock serum) among the ultracentrifugal partitions of the sera of laying hens, the sera of cocks, and the yolks are tabulated in Table I. LSI and SCI were yellow or white, LSII and CSII were the color of serum, and contained most parts of the total serum. LSIII and CSIII were a sediment and soluble in isotonic saline. YI was a clear yellow supernatant and soluble in isotonic saline. YII was a white sediment of yolk and insoluble in isotonic saline, but soluble in hypertonic (more than 8%) saline. Concentration of lipid P was very high in LSI and low in LSII and LSIII. Almost all protein P was sedimented in LSIII, and none was found in LSI. Concentration of nitrogen in LSI was low, and very high in LSIII. Antigenic potency with anti-laying hen serum absorbed with cock serum was high in LSI and LSIII and very low in LSII. Antigenic potency per unit protein was highest in LSI, high in LSIII and lowest in LSII. In the serum of cocks, concentration of lipid P was low compared with that in the serum of laying hens, and no lipid P was found in CSI. No protein P was found in the serum of cocks and immunologic reaction with antiserum was negative. Concentration of lipid P was high in YI and low in YII. Protein P was almost completely sedimented in YII and was scarcely found in YI. Concentration of nitrogen in YII was twice as high as in YI. Antigenic potency with antiserum was higher in YII though high potency was still found in YI. Antigenic potency per unit protein was twice as high in YII as in YI.

Electropherograms and radioautograms of the serum of laying hens and antigenic potency of each fraction are illustrated in Fig. 1. In the electrophoretic pattern of LSI only one slow moving component was found. Staining with Sudan III revealed it was a lipoprotein. The strong antigenic potency was noted in this low-density lipoprotein, which is considered a phospholipo-P-free protein. This specific antigenic substance in this reaction was completely destroyed by incubation at 80°C for 30 min. LSII contained a large amount of albumin. Antigenic potency was not found in any fractions

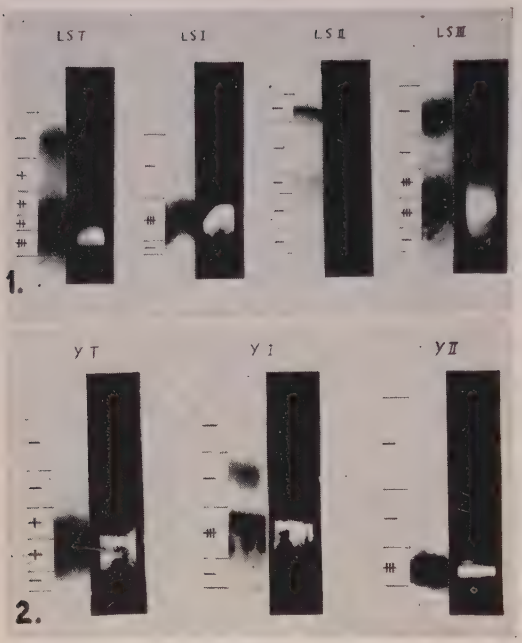

FIG. 1. Electropherograms of serum of laying hen (LST) and its ultracentrifuged partitions (LSI, LSII, LSIII), these radioautographs, and antigenic potency with anti-laying hens serum absorbed with cock serum shown by —, +, ++, and +++, of each fraction of electropherograms.

FIG. 2. Electropherograms of serum of the laying hen (YI) and its ultracentrifuged partitions (YI, YII), these radioautographs, and antigenic potency with anti-laying hen serum absorbed with cock serum, shown by —, +, ++, and +++, of each fraction on electropherograms.

of LSII. LSIII contained at least 3 components; albumin, gamma globulin and phosphoprotein. The positive antigenic reaction was found in a wide range of slow moving components. This specific antigenic substance(s) was also completely destroyed at 80°C. Electropherograms of the partitions of the serum of cocks obtained by ultracentrifugation, showed a complete absence of the lipoprotein component in CSI and absence of one of the slow-moving components, phosphoprotein, in CSIII. Electropherograms and radioautograms of yolk and antigenic potency of each fraction are shown in Fig. 2. YI contained 3 components. The slow moving one of these components was phospholipo-P-free protein, as shown by the stain with Sudan III and the radioautograph with P^{32}. The strong antigenic potency was found in this component. YII contained phosphoprotein which gave a positive reaction with anti-laying hen serum absorbed with cock serum. These specific antigenic substances in both fractions were completely destroyed at 80°C.

Results of the interfacial precipitin reaction tests with various antisera absorbed with cock serum are given in Table II. Antigenic titer with anti-LSI serum was high in LSI and YI, but negligible in LSIII and YII. The titer with anti-LSIII serum was high in LSIII and YII, but negligible in LSI and YI. While anti-LSIII, anti-YI or anti-YII serum reacted with all antigens, anti-Y serum reacted stronger with LSI and YI than with the other partitions, and anti-YII serum reacted stronger with LSIII and YII. In cross absorption test, the antibodies contained in anti-LSI serum and anti-YI serum were not absorbed with partition of LSIII and the antibody to LSIII was not absorbed with either LSI or YI. The residual antigenic potency to homologous antigen was still observed after the absorption with heterologous antigen. When anti-YI serum and anti-LSI serum were absorbed with YII, no precipitin reaction was observed.

Results of agar-antiserum gel single diffusion tests are illustrated in Fig. 3. When antigen LSI and YI were placed upon the gel prepared by mixing anti-LSI serum, a strong positive precipitin zone was observed, and a faint zone was observed in the case of antigen YII. This weak zone disappeared when the antiserum was absorbed with LSIII, while the stronger zone remained. No appreciable precipitin zone was observed in the gel with anti-LSII serum, though very faint

TABLE II. Precipitin Reaction between Partitions of Serum of Laying Hens and Yolk by Ultracentrifugation and Antisera to These Partitions.

Antiserum* to partition	Precipitin titer *vs* antigen				
	LSI	LSII	LSIII	YI	YII
LSI	5,120†	0	0	10,240	0
LSII	5,120	80	10,240	5,120	20,480
LSIII	0	0	10,240	0	40,960
YI	5,120	160	2,560	10,240	5,120
YII	1,280	80	10,240	320	40,960

* Absorbed with cock serum, and diluted 1:3.
† Expressed as the highest dilution of antigen giving a positive reaction with each antiserum.

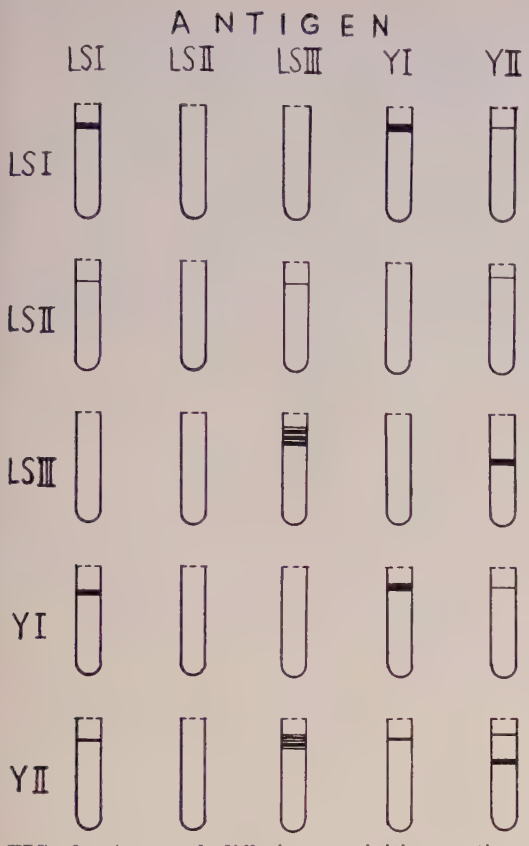

FIG. 3. Agar gel diffusion precipitin reactions among partitions of serum of laying hens and of yolk obtained by ultracentrifugation, and antisera to those substances. – – – boundary between antigen and agar-antiserum gel. ▬ strong perecipitin zone; —— weak one.

reaction was still found in cases of antigens LSI, LSIII and YII. Several zones were observed in the gel containing anti-LSIII serum when LSIII was used as antigen, but only one zone was observed when antigen YII was used. These zones were still observed after the antiserum was absorbed with LSI. The precipitin zone in the gel containing anti-YI serum was similar to that in the gel containing anti-LSI serum. When anti-YI serum absorbed with YII was used in a gel, no precipitin zone was found with antigen YI or LSI. In the reaction of anti-YII serum against each antigen, a zone was observed in both case of antigen LSI and YI, which disappeared when anti-YII serum was absorbed with YI, and 2 zones were found in the case of antigen YII, one of which disappeared when anti-YII serum was absorbed with YI. As antiserum to YII reacts with almost all the partitions of the serum and yolk except LSII and the immunologic reaction completely disappeared when the antisera to any partitions were absorbed with YII, YII may consist of at least 2 antigenic substances, one of which is also present in YI.

Discussion. The present work demonstrates that at least 2 specific antigenic substances are present in the serum of laying hens. They react with the antiserum to laying hen serum absorbed with cock serum prepared so as to react only with the serum of laying hens, the serum of estrogenized cockerels and pullets, or a solution of egg yolk. One of these substances is a low density phospholipo-P-free protein which is present in the top layer obtained by ultracentrifugation, the other is a phosphoprotein or a phosphoprotein complex which is present in the sediment obtained by ultracentrifugation. Similarly, 2 specific substances are present in egg yolk, one in the supernatant solution and the other in the sediment obtained by ultracentrifugation. These 2 specific substances are present in the serum of laying hens and are not found in the serum of cocks, and are capable of producing the specific antibodies by immunizing rabbits. The specific antibody or antibodies to one specific substance can not be absorbed with the other substance.

The present work also shows that approximately 90% of the phospholipides are not associated with phosphoprotein either in the serum of laying hens or in the yolk. A similar result was noted by Schmidt *et al.*(15) in the egg yolk. Low density phospholipo-P-free protein in the top layer of the serum of laying hens may be almost identical with that in the supernatant solution of yolk as shown by electrophoresis, N/P ratios, solubility in saline, and pattern of precipitin zone in agar-antiserum gel. This material may be a single substance because only one precipitin zone was found in the reaction. Phosphoprotein or phosphoprotein complex contained in the sediment of egg yolk is quite insoluble in isotonic saline and soluble in hypertonic

(more than 8%) saline, differing from that contained in the serum of laying hens which is soluble in isotonic saline. The N/P ratio in the sediment of the serum of laying hens is much higher than that in the sediment of egg yolk. Furthermore, the antigenic substances in the sediment of laying hens are not a single substance, because, when antiserum to the sediment of the serum of laying hens was used, several precipitin zones formed with the sediment of the serum, but only one zone formed with the sediment of yolk. These results show a difference between the chemical structure of phosphoprotein or phosphoprotein complex of the serum of laying hens and that of egg yolk. The substance in the blood of laying hen may be transformed into substance in egg yolk during the passage through the granulosa of the follicle.

Summary. Electrophoretic and immunologic studies on the partitions of the sera of laying hens and of cocks and egg yolk obtained by ultracentrifugation showed the presence of 2 specialized components, (a low density phospholipo-P-free protein and a phosphoprotein or a phosphoprotein complex) either in the serum of laying hens or in egg yolk, which were not found in the serum of cocks. Each component was capable of producing the specific antibody or antibodies. The specific antibody or antibodies to one specialized component could not be exhausted by absorption with the other component of the serum. Low density phospholipo-P-free protein, found in the top layer of the serum of laying hens, obtained by ultracentrifugation is almost identical with that found in the supernatant solution of egg yolk obtained by ultracentrifugation. This protein is a single antigenic substance as shown by the appearance of only one precipitin zone in the agar-antiserum gel. Phosphoprotein or phosphoprotein complex, found in the sediment of the serum of laying hens, is not identical with that found in the sediment of egg yolk, since the former is soluble in isotonic saline, while the latter is quite insoluble in isotonic saline. The pattern of precipitin reaction zone to the material in sediment of serum in agar-antiserum gel, which showed several zones, differed from that to the material in sediment of yolk.

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Effects of Vasopressin on Femoral Arterial Blood Flow and Pressure in the Anesthetized Dog. (26386)

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The action of extracts of the posterior pituitary on renal reabsorption of water and electrolytes, milk let-down, and uterine activity has been stressed in studies of the physiological role of this endocrine gland. Little evidence is available regarding the action of exogenous vasopressin on the cardiovascular system, with the exception of studies in which the dose employed greatly exceeds the maximal antidiuretic dose(1,2,3); recently, Kitchin(4) has studied the effects of small doses of vasopressin on hand and forearm blood flow. The present study considers the dose-response relationship between vasopressin and femoral arterial blood flow and pressure.

Materials and methods. Five mongrel dogs weighing 9.5 to 14.6 kg were used. Sodium pentobarbital, 30 mg/kg, was used to induce anesthesia; supplementary doses were administered during the experiment to maintain a moderately deep surgical plane. Mepesulfate* or heparin was administered after completion of all dissections and before cannulation to prevent coagulation. The right jugular vein was cannulated percutaneously by means of a plastic needle described by Lundy (5); supplementary anesthesia, anticoagulant, and intravenous vasopressin were administered through it. Rate of flow in the left femoral artery was measured with a cannulating-type square wave electromagnetic flowmeter after the design of Denison and Spencer(6). The artery was cannulated proximally and distally through a single slit with polyethylene tubing (PE 280). A shunt around the flowmeter pickup allowed the zero flow to be established without producing occlusion of the arterial inflow to the bed under study. This shunt was occluded during the experiment. A short length of rubber tubing, inserted into the distal cannula, was used for intra-arterial injections. Lateral pressure was recorded distal to the flowmeter pickup with a Statham pressure transducer. The output of the flowmeter and pressure transducer was fed into a 2-channel Grass polygraph equipped with low-level D.C. preamplifiers. The flowmeter was calibrated at the end of each experiment by bleeding the animal through the pickup into a graduated cylinder at different rates.

Vasopressin† was injected intra-arterially (IA) in graded doses of 0.1 to 5.0 milliunits (mU) of pressor activity; intravenous (IV) doses of 5 to 100 mU were also studied. Dilutions were prepared for each experiment in 0.9% saline; IA injections were limited to 0.2 ml and I.V. to 3 ml so that they could be administered rapidly, *i.e.*, <2 seconds. Con-

TABLE I. Mean Response ± Standard Error Following IA Injection of Vasopressin Expressed as Per Cent of Control Flow, Pressure, and PRU.

Dose, mU	No. of animals*	Time, sec.	Flow, %†	Pressure, %	PRU, %†
.1	4 (5)	50 ± 5	93 ± .9	101 ± .3	108 ± 1
.2	4 (7)	49 ± 7	89 ± 1.4	101 ± .6	113 ± 2
.5	4 (6)	64 ± 4	81 ± 2.8	101 ± 4.8	126 ± 5
1.0	5 (6)	47 ± 7	68 ± 1.8	101 ± 2.0	149 ± 5
2.0	5 (6)	52 ± 9	60 ± 4.9	102 ± 1.7	176 ± 18
5.0	4 (4)	49 ± 11	36 ± 5.3	104 ± 1.0‡	306 ± 41

* No. of injections in parentheses.
† Flow and PR changes significantly different from control ($p < .01$).
‡ Significantly different from control ($p < .05$).

* Hoffmann LaRoche, Inc., Nutley, N. J.
† Pitressin,(R) obtained from Parke, Davis & Co.

trol saline injections were made by both routes in each experiment. A minimum of 4 minutes elapsed between subsequent injections.

Changes in mean pressure and flow were calculated at the point of greatest response, whether it represented a decrease or increase, and are expressed as percentage of control value. Resistance to flow was calculated in PRU(7) and expressed as percentage of control value.

Results. Control data. IA and IV saline did not produce a significant change in flow or pressure. The 2-minute period before each injection was used to establish the control flow and pressure for that injection.

Intra-arterial injections (Table I). The flow was observed to decrease following each of the 34 injections by this route; mean decrease at time of maximum response was found to be significantly different from the control ($p<0.01$) at all doses. Mean arterial pressure remained essentially constant during the entire flow response.

Intravenous injections (Table II). A biphasic flow response was observed after administration by this route; an initial increase in flow which had a duration of 40-180 seconds followed by a decrease in flow below the control level. In general, mean pressure change at either the maximum increase or maximum decrease in flow could not be interpreted as significant. Maximum mean increase in pressure observed during the entire response was significantly different from the control ($p<0.05$), although only 5 mU fforteatohmrcmx 50 mU vrs 100 mU show significant differences from each other.

Discussion. Our results indicate that vasopressin administered IA induces vasoconstriction without significant elevation of arterial pressure; a consistent dose-response relationship was found. Kitchin(4) observed a similar reduction in forearm flow during IA infusion of vasopressin. The transient increase in flow preceding vasoconstriction reported by Woodbury and Ahlquist(2) was not observed in any of our experiments. They attributed this increase to a dilation

TABLE II. Mean Response ± Standard Error (S.E.) Following I.V. Injections of Vasopressin Expressed as Per Cent of Control Flow and Pressure.

Dose, mU	No. of animals*	Maximum flow increase			T_c, sec.†	Maximum flow decrease			Maximum pressure	
		Time, sec.	Flow, %	Pressure, %		Time, sec.	Flow, %	Pressure, %	Time, sec.	Pressure, %
5	4 (6)	53 ± 11	114 ± 3.9	100 ± .4	116 ± 27	214 ± 39	90.8 ± 2.1	101 ± 1.1	57 ± 16	103 ± .5‡
10	5 (6)	42 ± 7	128 ± 5.6	103 ± 2.3	93 ± 16	183 ± 20	86.2 ± 2.0	105 ± 2.0‡	73 ± 22	106 ± 1.9‡
25	4 (4)	41 ± 8	134 ± 9.4	107 ± .5‡	106 ± 22	234 ± 25	80.8 ± 2.0	103 ± 1.6	82 ± 35	104 ± 1.4‡
50	5 (5)	35 ± 4	144 ± 8.2	102 ± 1.3	97 ± 21	186 ± 29	65.7 ± 4.6	105 ± 1.5‡	103 ± 29	108 ± 1.0‡
100	3 (3)	38 ± 5	163 ±21	103 ± 0 ‡	64 ± 11	143 ± 35	66.7 ± 2.8	108 ± 5.7	83 ± 6	112 ± 3.2‡

* No. of injections in parentheses.
† Time to return to control flow following initial increase.
‡ Pressure changes significantly different from control ($p<.05$).

of the cutaneous beds supplied by the femoral artery. Experiments are in progress to study the comparative effects of vasopressin on cutaneous and muscular flow.

A consistent dose-response relationship for flow was noted following IV administration both at the time of maximum flow increase and decrease; in the latter case, saturation seems to occur at higher doses. This biphasic response was also observed by Woodbury and Ahlquist(2), who used considerably larger doses than we employed. The initial increase in flow probably results from the slight elevation of arterial pressure; the inconsistent dose-response relationship for pressure suggests that other factors may also be important. It was therefore postulated that reflex adjustment of the peripheral resistance was necessary to explain fully the initial increase in flow.

The secondary decrease in flow results primarily from the direct action of vasopressin on the peripheral bed. This interpretation considers the time fatcor as well as an estimate of the effective concentration of the drug in the periphery. It appears unlikely that this vasoconstriction occurs reflexly since a moderate elevation of pressure was observed to prevail before and during the secondary flow response.

It has been repeatedly demonstrated that small amounts (0.25 mU/kg) of exogenous vasopressin produce a pronounced antidiuretic response in the dog(8). It has been argued that vasopressin is not important physiologically in control of cardiovascular function in that relatively large quantities (200 mU) are required to elevate the arterial pressure of the dog(8), and that significant reduction in arterial pressure has not been reported following hypophysectomy. The results of the present study indicate that elevation of arterial pressure is not an adequate criterion for assessing vascular activity of vasopressin whereas the elevation of peripheral resistance reveals the sensitivity of the peripheral bed. It is tentatively postulated that submaximal antidiuretic amounts of the exogenous material are really vasoactive amounts. This hypothesis is currently under investigation.

Conclusions. The dose-response relationships which describe the effects of small amounts of vasopressin on femoral arterial pressure and flow have been presented. The IA and IV routes of administration were examined in 5 experiments on anesthetized dogs. The decrease in flow following IA administration and the secondary decrease in flow following IV administration have been attributed to vasoconstriction in the vascular bed supplied by the femoral artery. The primary increase in flow following IV administration has been discussed in terms of elevation of central arterial pressure and reflex vasomotor activity. It is postulated that submaximal antidiuretic amounts of exogenous vasopressin are vasoactive.

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Effects of Varying Rates of Sodium Excretion on Na^{22} Kinetics.* (26387)

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The present investigation was undertaken to ascertain the feasibility of estimating daily total exchangeable sodium with Na^{22} as part of an experiment involving bioassay of diuretic agents(1). Threefoot, Burch and Reaser (2) studied the serum and urinary biological decay curves of Na^{22} in control subjects and in patients with congestive heart failure and the nephrotic syndrome. They found that the presence of edema fluid, variation in sodium intake and administration of mercurial diuretics and desoxycorticosterone acetate (DCA) influenced the slope of these curves. Under similar conditions we have calculated daily total exchangeable sodium and serum specific activity and have compared their technic with the chemical measurement of serum and urinary sodium.

Materials and methods. Eleven male patients from the medical wards of the Philadelphia General Hospital were selected for this study. Eight of these patients had well-controlled diabetes mellitus, and were selected because of the better facilities for accurate urine collections and dietary supervision which were available on their ward. Nine patients showed no clinical evidence of abnormal fluid retention; one had edema due to the nephrotic syndrome and another congestive heart failure.

Caloric intake was maintained at a constant level in the individual patient and ranged from 1945 to 3370 calories. Sodium intake was *ad libitum* but appeared to be fairly constant for individual subjects as judged by the small fluctuations in daily urinary sodium excretion during the control periods. After an initial control period of one week, sodium chloride (178 to 258 meq Na), DCA 30 mg and hydrochlorothiazide, 150 mg, were administered individually or in combinations daily for 3 to 7 days. Subsequent control periods between administration of the above agents were 3 to 7 days duration.

Twenty microcuries of Na^{22} were given intravenously to each patient and 24 hour urine collections were obtained daily for 10 to 48 days. Serum and urine samples were analyzed daily for sodium with a flame photometer and for Na^{22} radioactivity with a well type scintillation counter. The cumulative urinary excretion of Na^{22}, which was ex-

TABLE I. Characteristics of Patients.

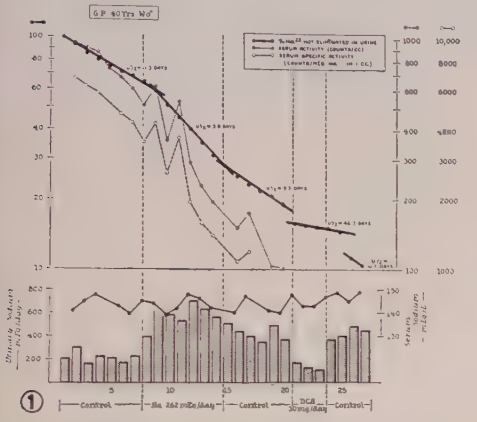
Case No.	Age	Body wt, kg	Na_E, meq	Na_E/kg body wt	Na^{22} U½, days	Na^{22} S.A.½, days	Control Na excret., meq/day	Avg % change daily in Na_E over 1st week
1	55	52.0	3043	58.5	9.4	6.8	227 ± 44‡	+4.1
2	40	56.2	3361	59.8	11.3	6.6	213 ± 44	+4.5
3	63	75.9	2995	39.3	19.3	17.5	131 ± 29	+ .4
4	63	70.5	3088	43.8	13.0	11.7	197 ± 43	+ .5
5	59	57.0	3113	54.6	13.0	8.1	186 ± 57	+2.0
6	65	75.1	3877	51.6	23.5	12.1	130 ± 42	+1.9
7	56	69.3	3519	50.8	20.8	8.8	135 ± 11	+3.4
8*	40	74.1	3135	40.7	31.7	30.6	79 ± 18	+ .1
9†	55	75.2	3240	43.1	73.4	167.0	20 ± 3	− .1
10	63					13.5		
11	56					26.2		

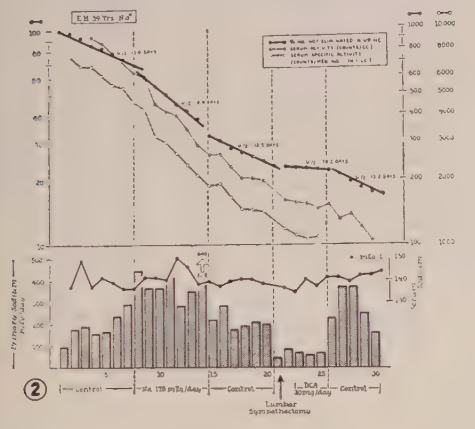
* Nephrotic syndrome. † Congestive heart failure. ‡ Mean ± S.D.

* This work was aided by grant from U. S. Public Health Service.

Desoxycorticosterone acetate was supplied through the courtesy of James Smith, Ciba, and hydrochlorothiazide through Merck, Sharp & Dohme.

pressed in terms of per cent of injected Na^{22} was plotted as an exponential function according to the method of Threefoot, Burch and Reaser(2). Serum radioactivity (counts per minute per ml serum) and serum specific activity (S.A.) (serum radioactivity per meq sodium per ml serum) were plotted as the fraction of the administered dose against time on a semi-logarithmic graph. Total exchangeable sodium (Na_E) was calculated daily by dividing the specific activity of the serum into the amount of Na^{22} present in the body, using as an approximation the assumption that all excretion of radioactivity occurred *via* the urine(2). U ½ was defined as the time in days required to eliminate 50% of the administered radioactivity in the urine. S.A. ½ was defined as the time in days to eliminate 50% of the serum specific activity after equilibrium distribution.





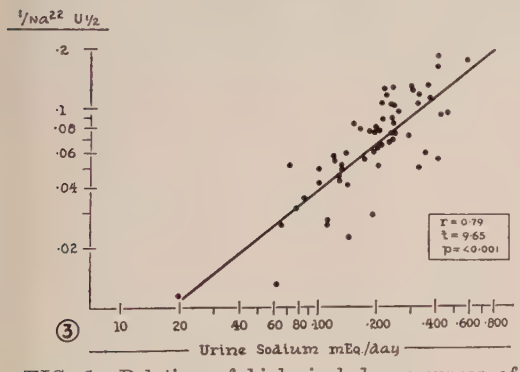


FIG. 1. Relation of biological decay curves of Na^{22} to serum and urinary sodium in case 2.

FIG. 2. Relation of biological decay curves of Na^{22} to serum and urinary sodium in case 5.

FIG. 3. Urinary sodium excretion and $1/Na^{22}U½$ plotted on a logarithmic scale.

Results. In Table I are summarized data obtained in these experiments. As has been noted previously(2), the $Na^{22}U½$ is usually greater than the S.A. ½. In 4 patients, initial Na_E was normal, in 5 it was elevated (normal range, 32 to 49 meq per kg body weight(3). Calculation of Na_E during the first week showed an average daily increase of +1.9% (range, –0.1 to +4.5%). Thereafter, acute changes in sodium excretion produced by various agents were not generally reflected by trends in Na_E.

Biological decay curves, serum sodium and urinary sodium are depicted for 2 patients (Fig. 1, 2). Slopes derived from the curves of urinary excretion clearly reflected the changes in sodium excretion. Large doses of sodium chloride or diuretic therapy which increased sodium excretion steepened the slopes, while administration of DCA promoted sodium retention and flattened the slopes. Serum radioactivity concentration and specific activity paralleled each other. These parameters were quite variable over short periods so that when sodium excretion was altered by drugs the slopes could not be derived. Initial observations suggested that a rise in the slope of serum specific activity above the control slope might occur with DCA administration. For this reason, DCA was given to subjects 10 and 11 while serum radioactivity was high, *i.e.*, after 7 days, but the above response was not confirmed.

The reciprocal of the derived U½ was plotted against sodium excretion for the same periods. There was good correlation,

$r = 0.79$ (Fig. 3). A slightly higher correlation, $r = 0.90$, was obtained when the test period was divided by the control period immediately preceding it. Radioactivity in the urine samples diminished to such low levels after 30 days that reliable slopes could not be constructed.

Discussion. Daily estimation of total exchangeable sodium by a single injection of Na^{22} over prolonged periods would be a welcome addition to our investigative armamentarium. Unfortunately, the progressive rise in Na_E, coupled with inability to correlate levels of Na_E with acute changes in sodium excretion makes this approach impractical. Compared to our finding of an average daily increase of 1.9% in Na_E, Miller(4) *et al.* found a 1.0% increase over a week's period in 4 subjects maintained on a low salt diet. Since external counts over the patella remained constant while serum specific activity diminished, they postulated that this increase in Na_E might be due to increasing exchange of Na^{22} with bone sodium. However, other causes which might account for the rise in Na_E include extrarenal loss of sodium, calculation of serum specific activity when equilibrium has not been attained, and errors in urine volume measurements, all of which may lead to large cumulative errors.

The biological decay curves of serum Na^{22} radioactivity and specific activity paralleled each other. Because of wide daily fluctuations, these curves were not very valuable in depicting alterations of sodium excretion during the short periods used in this study. On the other hand, the cumulative urinary excretion curves of Na^{22} showed good correlation with daily sodium excretion. However, no additional information was provided by the former determination over the latter.

Summary. Biological decay curves of Na^{22} excretion showed good correlation with urinary sodium excretion during alteration of sodium excretion by sodium chloride, DCA and hydrochlorothiazide over periods of 3 to 7 days. During these short intervals, biological decay curves of serum radioactivity and serum specific activity were too variable to allow correlation with urinary sodium excretion. Daily total exchangeable sodium shows a progressive rise and cannot be used to reflect acute changes in sodium balance.

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Effect of Water Deprivation on Nasal Mucous Flow.* (26388)

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The combined action of mucous cells and ciliated cells to form a moving blanket which collects particles and transports them to given places for disposal is present throughout the invertebrate and vertebrate phyla(1). In the respiratory tract of vertebrates this mechanism functions to keep the surfaces free of debris. Most of our knowledge concerning its malfunction relates to the effects of noxious gases and other "external factors" (5). Initially through a chance observation, we have found that degree of systemic hydration of herring gulls and chickens has a marked effect on movement of the mucous

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† With technical assistance of Jean Ramsey Morris.

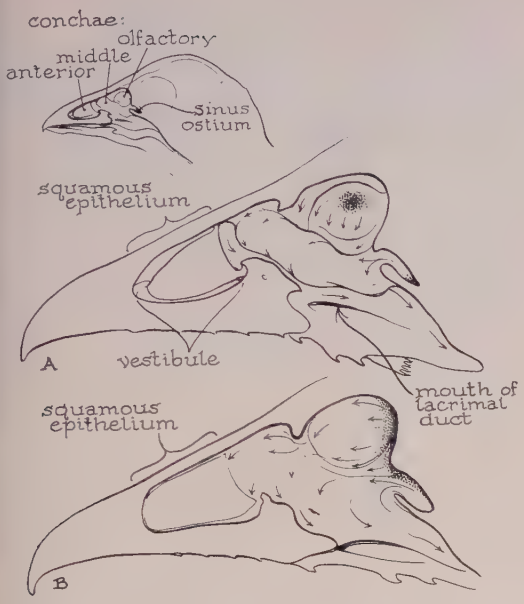

FIG. 1. Structures involved in nasal mucus flow in the chicken as seen in sagittal section. A: Lateral wall. B: Septum. Unless the mucosa is damaged in sectioning, or unless bits of debris such as bone chips or feathers fall onto it, the ink-carrying blanket sweeps antero-radially off the olfactory concha (A) moving as a cohesive blanket yet proceeding fanwise across entire length of the middle concha so that the anteriorly directed upper portion may go along the roof margin to the base of the nasal vestibule, thence ventrally to flow backward along the floor into the choana, joined at the mouth of lacrimal duct by the blanket lining that duct. The main body of the blanket flows over the convexity of the middle concha and inside its scroll, to be funnelled into the pharyngeal current at the base of the concha, joining secretions which have come directly ventrally from the lateral wall and from the maxillary sinus. Septal blanket (B) flows rapidly forward to fan out as shown in diagram. The main portion curves ventrally and then sharply back toward the choanae, while a narrow but strong current continues along roof area to vestibule, where in the living bird it presumably spills onto the non-ciliated squamous lining and out the nares.

blanket in the upper respiratory tract of these birds.

Rate and pattern of flow of the blanket of mucus within the nasal chambers of wild-caught subadult Herring Gulls, *Larus argentatus*, were observed on freshly killed birds during the summer at Marine Biological Laboratory, Woods Hole, Mass. They were anesthetized with Nembutal, the upper skulls were sagittally sectioned so that the septum was intact on one half and the conchal structures on the other, and an even sheet of colloidal India ink diluted with normal saline (2 parts ink, 1 part saline), was spread on the posterior limit of the septal mucosa, on the rim of the maxillary sinus ostium, and on the medial surface of the olfactory concha. Motion of the blanket could then be observed in the dissecting microscope as the foci of ink were incorporated into it and were swept along the established ciliary pathways (see Fig. 1 and accompanying text).

Two gulls in succession were found to have such flaccid veins that intravenous anesthesia could be accomplished only after much frustration. When ink was placed on the mucosae of these birds in the usual way, it lay immobile. The mucosal surfaces were coated with thick glassy mucus, there was no visible beating of cilia, and a few thin wisps of ink slowly sank downward through the viscous coat to puddle on the mucosa. Enquiry revealed that these 2 gulls were from a group that came to our attention some time after they had been brought in, due to an unintended delay in notification. Gulls used for investigation at the Laboratory are caught by hand at their island breeding site (a procedure which involves a rigorous chase and considerable emotional excitement on the part of the birds), secured in burlap bags, and brought to the mainland where they are maintained in outdoor cages until required. Most of them are large immature birds not quite able to fly but nearly as large as their volant mates. We habitually used them as soon as they were brought in. This group, then, were not only disturbed as a result of the trapping experience but were dehydrated as well. The remaining gulls in the group were given water, and later the same evening 2 of them were tested to check the result of rehydration. The movement of the blanket appeared normal in both.

The next "catch" of gulls was the last of the season. Of 4 birds of nearly equal sizes, 2 were deprived of water for 12 hours after being brought in, and 2 were fed and watered in the usual way. Of the water-deprived birds, the nasal mucous blanket moved very sluggishly in one and essentially not at all in the other. No ciliary motion was visible

through the thick coats of mucus, but when bits of mucosa were stripped off and observed in the phase microscope, ciliary beating was plainly seen. There was swift movement in the 2 control gulls over a healthy mucosa shimmering with rapidly beating cilia. The mean rate of motion of the blanket in all of the normally nourished birds was about 10 mm/minute.

On returning to Baltimore, a flock of 8-week-old White Leghorn chickens was divided into experimental and control groups, the latter being fed and watered in the routine way. Experimental birds were deprived of water, but not their dry mash feed, for 4, 16, 24, 36, 48, and 72 hours; 4 birds were in each deprived group, each of which had 2 concomitant controls, and 2 additional birds were included in the 72 hour group in order to observe the effect of rehydration. Using the same procedure described for the gulls, at 4 hours there was no apparent change in rate of mucus transportation; at 16 hours there was noticeable sluggishness in various areas in each of the deprived birds but not in the same area in each bird. At 24 hours there was essentially no motion of the blanket in 3 birds and fair but erratic motion in the fourth. At 36 and at 48 hours motion of the blanket was uneven throughout, and mean rate in any area did not exceed one millimeter a minute. At 72 hours the pattern of flow was again uneven throughout, but along one particular pathway that was consistent for all 4 birds it moved at a mean rate of nearly a millimeter a minute. This was along the upper lateral wall of the olfactory chamber. The pull of the ink-laden areas in all of the birds deprived for 16 hours or more was characterized by a sluggish hesitancy quite unlike the smooth swift flow of a healthy mucus-ciliary current. Control birds in each group showed this normal flow, rate of which in the chickens as in the gulls was an average 10 mm/minute. The 2 chickens which had been deprived of water for 72 hours were rehydrated, then killed after 36 and 53 minutes respectively. Motion of the mucous blanket was apparently normal in both rate and pattern of flow on both birds; no electrolytes were added to the water of rehydration.

Brisk ciliary motion could be seen at all times on all of the deprived as well as on all of the control chickens. None of these at any phase showed the degree of viscous glazing observed in the dehydrated gulls, though some thickening, puddling, stickiness, and glassiness was visible grossly on most of the deprived birds. It may be that the degree of dehydration was greater in the gulls. Both gulls and chickens were treated with ink within less than 90 seconds after decapitation. All observations were at room temperature and no attempt was made to control humidity.

The mechanism by which mucus remains in position as though anchored to the mucosa, while cilia continue to beat, is not understood. A clue may have been supplied by some current studies of whole mounts and sections of nasal organs of dehydrated baby chicks in which the mucous sheet was fixed *in situ*. Groups of chicks were deprived of water for 24, 48, and 72 hours. One or 2 from each group showed very thick, glassy, bubbly mucus. From each group, some were used for study of ink transportation, some were fixed for histology, and some were prepared according to Moe's method(2) which allows direct examination of surface mucous glands in whole mounts. Mucus was fixed *in situ;* whole septa and whole conchae were dissected out, stained with periodic acid-Schiff reagent, cleared, and viewed at 400× magnification by transmitted light. Fig. 3 shows the altered appearance of the surface glands in a whole mount of a dehydrated chick middle concha, and threads of mucus may be seen adhering to the surface. The threads show also in the histological section through the concha. The mucous blanket was apparently fixed while in the act of tugging toward the direction of ciliary propulsion, but was anchored to the mucosa by fine strands stretching tautly from the surface to the blanket. Such strands may evidently stretch until they snap "like a rubber band," as Proetz has described strands of mucus in another situation(3).

Some of the kinds of anchoring, stretching,

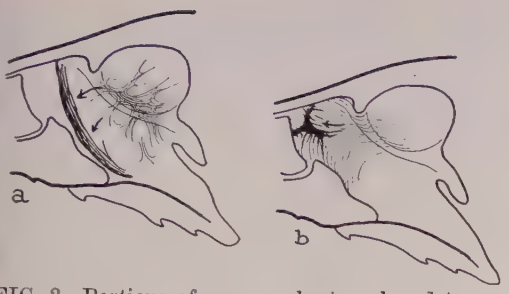

FIG. 2. Portions of mucous sheet anchored to mucosa in whole mounts of dehydrated chicks. a. Septum of a chick water-deprived for 24 hr, showing matted mucus adherent to inner rim of olfactory impression and mid-septum, and a ridge of mucus which had apparently begun to pile up along the forward line of advance of this portion of the sheet. Threads of mucus hold the blanket attached to the surface. b. Septum of a chick water-deprived for 72 hr, showing a band of mucous blanket fixed at a time that it was apparently being propelled by the cilia while anchored to several rows of glands or cilia.

and piling up of mucus seen in these preparations are shown in Fig. 2. Septum "a" shows that a thin portion of the sheet may remain partly stuck while the remainder is carried forward; in this case the forward moving part then piled up in a ridge along the line of advance. Septum "b" shows a segment of blanket anchored to the neighboring rows of glands. The size of the strands is greatly exaggerated in the drawing; in reality they are exceedingly delicate. The fine lines in the diagram represent the pattern of lineation of the glands in the immediate area of the anchored portion of the sheet.

Lucas and Douglas(4) have described the mucous sheet as consisting of an outer viscous and an inner serous portion. A situation which seemed to act this concept was seen in one of the chicks which had been water deprived for 72 hours. When India ink was spread along the posterior rim of the lateral half of the chamber in the usual way, most of it lay immobile on the surface, and a few wisps sank down through the thick mucus and lay on the mucosal surface. Quite suddenly, one of these underlying small puddles began to streak forward underneath the still immobile surface, climbing over the curve of the maxillary concha in 8 or 9 parallel lines, the ink particles apparently propelled along contiguous rows of the cilia. Within a few seconds, the heavy over-lying sheet began slowly to move in the same direction, so that a deep and a surface burden of ink could clearly be seen moving simultaneously at different rates.

To think of the mucous sheet in terms of layers may not be as accurate as the concept of a continuing secretion of the mucous cells which is variable in character (thin and fluid, thick and viscous, stringy, or lumpy) and which overlies a separate fluid secretion.

Discussion. Environmental humidity of less than 50% has been shown to stop the activity of cilia in trachea of rats within 8 to 10 minutes(5). In several species of animals and in monkeys and man, it has been noted that the path of the principal air currents between nares and choanae is the main factor determining rate of ciliary beat: motion is more rapid in the sheltered areas, slower along the paths of rapid airflow(6). This is true also in gulls and chickens, a maximum rate consistently clearing the olfactory chambers and maxillary sinuses and a visibly slower rate obtaining on the mid-septum. It was especially apparent in the chickens that after deprivation the motion of the blanket, though decelerated, persisted in these sequestered areas after it had effectively halted on the mid-septum. Histological sections of chickens and gulls show that the glands in the olfactory border area and around the sinus ostium are much deeper than elsewhere in the chamber. Whether the mucous secretion is intrinsically different in given areas, or whether changes of external environment or internal water balance induce area-specific changes in viscosity is not known. Ciliary action is known to be less affected by certain kinds of environmental changes than is the mucous blanket, neither accelerating nor decelerating under conditions which retard, speed up, or immobilize the overlying blanket(5,6). That cilia continued to beat under immobilized mucus in water deprived gulls and chickens was perfectly clear. Acute and chronic dehydration are known to have severe effects on systemic and cellular fluid and electrolyte balances(7).

Although degree of dehydration might be

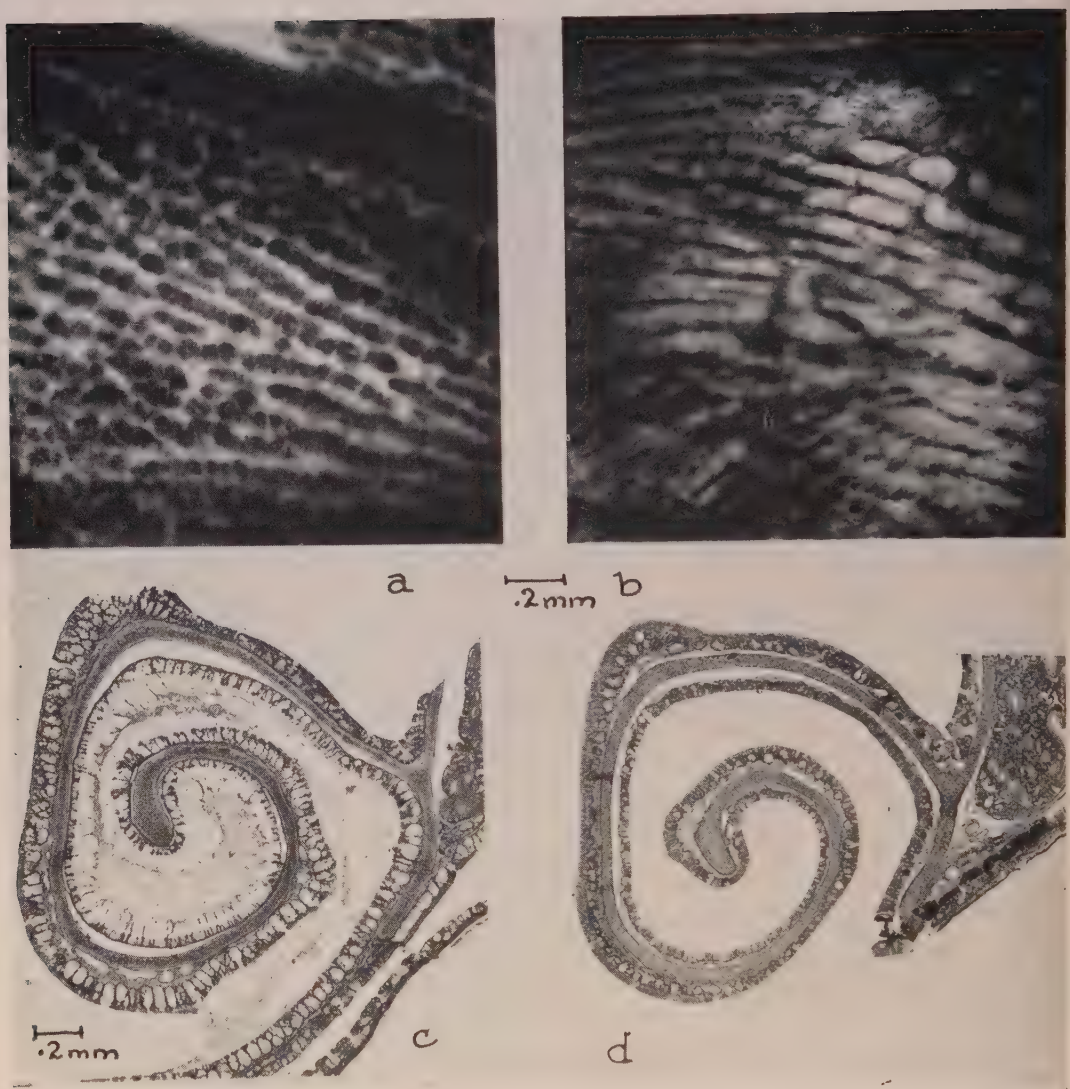

FIG. 3. Photographs of PAS-stained whole mounts showing inner base area of maxillary concha of: a. A normally hydrated chick. Mucus is in rows of glands. b. A chick deprived of water for 72 hr. Mucus does not show in glands, but seems held in or between rows. c. Section through the middle of middle concha of a chick deprived for 72 hr; threads of mucus stretch from mucous sheet to mucosa. H and E. d. Section through same area of a normally hydrated chick. H and E. Note difference in appearance of mucous glands in dehydrated and normal chick. Separation of mucosa from cartilage in "c" and "d" are artifacts of preparation.

expected to have a direct effect on viscosity of the mucus secreted, we have been unable to find references to the effect of internal hydration on the function of the respiratory tract. It is not surprising that water deprivation, alone or in combination with emotional stress, should be reflected in some way in production or transport of nasal mucus. It seems apparent in these crude but suggestive observations on herring gulls and chickens that systemic dehydration may have a pronounced effect on transportation of mucus in the upper respiratory tract. It has been reported that dehydration has less effect on pharyngeal mucous secretion in dogs and man than on parotid secretions(8).

Whether the nasal mucus of birds may be especially responsive to water deprivation is

not known, but preliminary experiments with 21-day-old mice indicate that the blanket motion is markedly inhibited in certain areas in these mammals after deprivation.

Summary. A striking depression in rate of flow of the nasal mucous blanket was observed in 4 water-deprived herring gulls, in contrast to 10 normally hydrated gulls. The observation was repeated in a series of 20 water-deprived and 10 normally hydrated chickens. In both species normal mean rate of flow was about 10 mm/minute. Two gulls and 2 chickens that were deprived then rehydrated showed a normal rate of motion of the blanket. Anchoring of the mucous sheet to the mucosal surface by fine strands of mucus was seen in whole mounts and histological sections of conchae of dehydrated baby chicks.

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Sensitivity to Estrogen of Uteri of Ovariectomized Mice in Relation to Age.* (26389)

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It has been reported(1,2) that sensitivity to gonadal hormones of accessory reproductive organs of gonadectomized male and female rats increases with advancing age reaching a peak at age when the animals, if intact, would have reached puberty. Sensitivity then decreased with age. The object of the present investigation was to determine sensitivity to estrogen of uteri of several strains of ovariectomized mice in relation to age.

Materials and methods. Forty mice selected from inbred strains and F_1 hybrids (A/Jax, C57BL/6, BALB/c, and BAF_1) served as intact controls to observe age at opening of vaginal orifice and at onset of estrous cycle.

Groups of mice from each strain were ovariectomized on day 21. A total dose of 0.5 μg estradiol benzoate in 0.1 ml sesame oil was divided into 2 equal portions which were injected subcutaneously at 24 and 16 hours prior to autopsy. Ovariectomized control mice were treated similarly with sesame oil alone. Autopsy was performed at 24, 34, 40, 46, 52, 58, 64, or 70 days of age. At autopsy uteri were weighed immediately after they were freed from mesometrium, vagina, and uterine tubes and were pressed gently between filter paper. Sensitivity to estrogen of uteri of ovariectomized mice were judged by percentage increase in weight of uteri above appropriate ovariectomized controls and by absolute uterine weight.

Results and discussion. Opening of vaginal orifice was found at about 37 days of age in A/Jax, C57BL/6, and BAF_1 mice and at about 28 days of age in BALB/c mice. Onset of estrous cycle in each of 4 strains of mice occurred about 10 days after opening of vaginal orifice.

Uteri of ovariectomized immature mice did not show prepubertal subnormal growth (Table I) as was seen in ovariectomized im-

* This investigation was supported by a research grant C-3108 from Nat. Inst. Health, P.H.S., U. S. Department of HEW.

TABLE I. Effect of 0.5 μg Estradiol Benzoate on Uterine Weight of Ovariectomized Mice.

Age, days	Treatment	A/Jax			BAF_1			C57BL/6			BALB/c		
		No. of mice	Uterine wt, mg*	PI	No. of mice	Uterine wt, mg*	PI	No. of mice	Uterine wt, mg*	PI	No. of mice	Uterine wt, mg*	PI
24	Estrogen	9	20.0 ±1.3	327.8	9	15.9 ±1.0	324.4	9	16.3 ±1.3	276.2	9	16.9 ±1.0	312.9
	Controls	6	6.1 ± .5		6	4.9 ± .4		6	5.9 ±1.1		6	5.4 ± .1	
34	Estrogen	8	13.1 ±1.3	285.0	8	11.6 ± .6	269.7	5	9.9 ±1.0	241.4	8	11.5 ± .4	280.4
	Controls	6	4.6 ± .1		6	4.3 ± .1		4	4.1 ± .7		9	4.1 ± .3	
40	Estrogen	8	13.9 ±1.3	278.0	8	8.1 ± .2	213.2	5	8.4 ±1.4	227.0	17	9.2 ± .4	235.9
	Controls	6	5.0 ± .1		5	3.8 ± .2		3	3.7 ± .2		10	3.9 ± .1	
46	Estrogen	8	12.0 ±1.0	244.9	7	9.4 ± .3	218.4	3	8.2 ± .6	227.7	5	10.0 ± .9	232.5
	Controls	6	4.9 ± .2		6	4.0 ± .4		3	3.6 ± .1		5	4.3 ± .3	
52	Estrogen	6	8.1 ±1.0	235.3	6	6.8 ± .4	188.8	5	6.9 ± .6	230.0	5	8.7 ±1.3	193.3
	Controls	4	3.4 ± .4		3	3.6 ± .1		3	3.0 ± .1		5	4.5 ± .1	
58	Estrogen	8	9.4 ± .3	229.6	8	7.7 ± .5	208.1	6	7.0 ± .7	226.0			
	Controls	6	4.1 ± .2		4	3.7 ± .6		4	3.1 ± .6				
64	Estrogen	4	10.2 ±1.0	261.5	5	6.7 ± .5	172.0	4	6.2 ± .8	221.4			
	Controls	5	3.9 ± .4		4	3.9 ± .5		4	2.8 ± .2				
70	Estrogen	9	9.0 ± .5	250.0	9	6.9 ± .1	202.9	4	6.1 ± .5	196.7			
	Controls	7	3.6 ± .1		6	3.4 ± .1		5	3.1 ± .2				

* Uterine weights are means and stand. errors. PI = % increase in uterine wt above appropriate control.

mature rats. Uterine weights of ovariectomized mice decreased with increase in age, probably due to the fact that estrogenic hormone had been secreted from the mice ovaries before these organs were removed on day 21.

Sensitivity to estrogen of uteri of ovariectomized immature mice did not increase at about the age of expected puberty in comparison to those of ovariectomized rats. The absolute uterine weights as well as the percentage increase in weights of estrogen treated mice uteri above those of the appropriate controls were less with increase in age (Table I). Species differences are probably a partial explanation for the contrasting results.

Summary. Uteri of ovariectomized immature mice did not show subnormal growth. No increase in uterine sensitivity to estrogen was observed at about the age of expected puberty in comparison to those of ovariectomized immature rats.

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Production of Congenital Malformations Using Tissue Antibodies. I. Kidney Antisera.* (26390)

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Embryologists have attempted to use tissue antibodies *in vitro* as growth inhibitors and disorganizers, and *in vivo* as teratogenic or abortogenic agents. There is evidence that specific and non-specific antigen-antibody reactions can cause fetal death or early resorption(1,2,3). Others have attempted to utilize organ or tissue antibodies as teratogenic agents specifically directed against that developing organ or tissue in order to produce specific congenital malformations(3, 4,5). The results of the latter experiment are somewhat controversial because of the low percentage of specific malformations produced by this technic.

Attempts to produce malformations in mice, using injected brain, have been reported to be successful(2,4), although no evidence was presented that anti-brain antibodies were developed or that they reached the fetus(2). Furthermore, the "malformations" of the central nervous system described were the same as those seen in fetuses dying or retarded from many noxious agents(2).

Lens malformations(3,5) were produced in mammals utilizing anti-lens antisera, but only in an extremely small percentage of cases (3 malformed eyes out of 611 fetuses in Miller's experiments). In any event, the reported instances of antibody teratogenesis would certainly classify antibodies as very ineffective teratogenic agents.

Methods. In our laboratory, we have been utilizing tissue antibodies for 3 years as an experimental embryological tool. Antigens were made from adult and fetal rat tissues: 21-day placenta, fetal liver, skin and brain; adult rat muscle, brain, kidney, liver, testes, plasma and red blood cells. The tissues were prepared by saline perfusion, homogenization and lyophilization, and stored in the deep freeze as a dry powder. Two injections of 200 mg of resuspended tissue powder were injected intraperitoneally into albino rabbits twice weekly, without any adjuvant, over a period of 3 months, followed by periodic bleeding. The presence of antibody was evaluated by agar diffusion plates and a serial dilution precipitin test.

Anti-kidney sera was obtained by multiple bleedings from 4 rabbits. Ten quantities of anti-kidney sera were obtained which gave reactions varying from very weak to extremely potent, as measured by number and density of bands in the agar diffusion plates, and the titers of the sera in the precipitin test.

Thirty-one pregnant rats were injected intravenously with .2 to 1.0 ml of rat kidney antisera on the 8th day of gestation. Number and location of the embryos were determined by laparotomy on the 8th day. At the 21st day of gestation, the mothers were sacrificed and fetuses were removed, fixed in buffered formalin and dissected.

The injection of teratogenic anti-kidney antibody was combined with the technic of vascular clamping of one horn of the pregnant uterus (Fig. 1g). The clamping technic was previously described and demonstrated that clamping the vascular supply to the pregnant uterus for one-half hour on the 9th gestational day produced insignificant gross changes in the fetus(6). The antiserum was injected intravenously just after isolating one horn of the uterus from the circulation, allowing the unclamped uterine horn to be in contact with the circulating kidney antiserum for the first one-half hour, while the clamped horn was isolated from the antiserum during this period. This technic enables one to evaluate the rapidity of action of embryonic growth inhibiting or malform-

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ing agents, since a greater effect on the unclamped side indicates that a significant effect has occurred during the period of clamping.

Gamma globulin was prepared from the anti-kidney plasma by the salting-out procedure using ammonium sulfate and reconstitution after dialysis with pH 7.4 borate buffer. The purified gamma globulin was labelled with I^{131} by the technic of Helmkamp *et al.*(7). Iodination was carried out utilizing 2 ml of gamma globulin and 1 mc of Na I^{131}. The labelled globulin was injected intravenously into pregnant and non-pregnant rats. From 12 to 172 hours later, the animals were anesthetized and perfused with 500 ml of saline. Samples of tissue (100-300 mg) were weighed and placed in 2 ml of 2 N NaOH and counted in a scintillation well counter.

Results. Fourteen of the 31 litters injected with antisera had quite severe malformations, consisting of anencephaly, anophthalmia, encephalocele, hydrocephalus, aplastic cerebral hemispheres, evisceration, chest wall defects, hypoplastic lungs, hypoplastic auricle, tear drop heart, right-sided aortic arch, fused kidneys, absent kidneys, absent ears, situs inversus, omphalocele, cleft lip, club feet and aplastic tail (Fig. 1a, b, c, d). Four samples of antisera produced 100% malformations if the dose was between .4 and .7 ml. Doses of .7-1.0 ml of potent antisera caused complete resorption. Growth retardation was frequently associated with malformation, although some malformations occurred in normal-sized fetuses at term. When maternal rat plasma was evaluated for presence of injected antibody following injection, none could be detected. This meant that either it reacted rapidly with the tissues, or the tests used in our laboratory were not sensitive enough to measure the antibody when it was diluted into the maternal rat circulation. In either event, teratogenic antibodies can be present in an organism and not be easily detected.

Injection of normal rabbit plasma, potent rabbit anti-rat plasma antisera and rabbit anti-rat erythrocyte antisera on the 8th gestational day served as control experiments. In spite of anaphylaxis, hemolytic anemia and apneic periods produced in the mother by the antisera, no congenital malformations were produced in the fetuses, and fetal weights were the same as non-injected animals that had laparotomies on the 8th day (Fig. 1e).

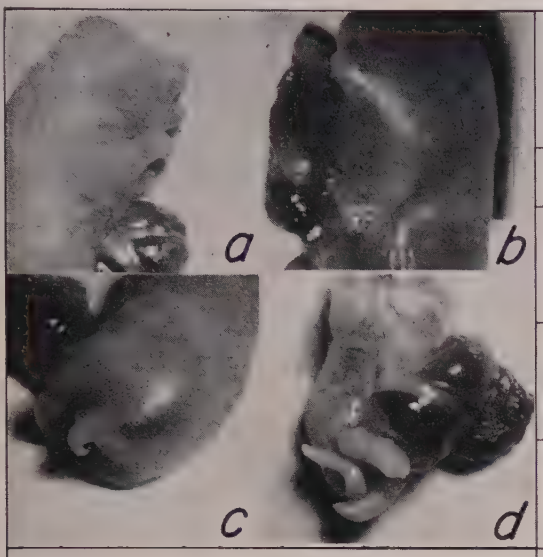
The animals that were injected with anti-kidney antiserum after clamping one horn of the uterus showed a higher incidence of malformations and fetal deaths on the unclamped side (Fig. 1g). Weights of the fetuses that survived on the unclamped side were significantly smaller than on the clamped side.

The localization of the I^{131} labelled anti-kidney serum confirmed previous reports of localization in the kidney, adrenal and spleen(8). In the pregnant animal, there was also localization within the placenta (Fig. 1f). The fetal placenta consistently localized a larger amount of the anti-kidney antibody than did the maternal placenta. The measured specificity of the anti-kidney serum for the placenta was diluted by the rapid growth of the placenta during the uptake studies. There were differential localizations in fetal tissues, but the quantitative amounts were insignificant compared to the labelled antibody in maternal kidney and fetal placenta (Fig. 1f).

Discussion. There is no doubt that some fraction of the anti-kidney antisera is teratogenic. This is not due to antibodies against red blood cells and/or sera trapped in the post-perfused kidney. There are several mechanisms by which the kidney antiserum may be teratogenic:

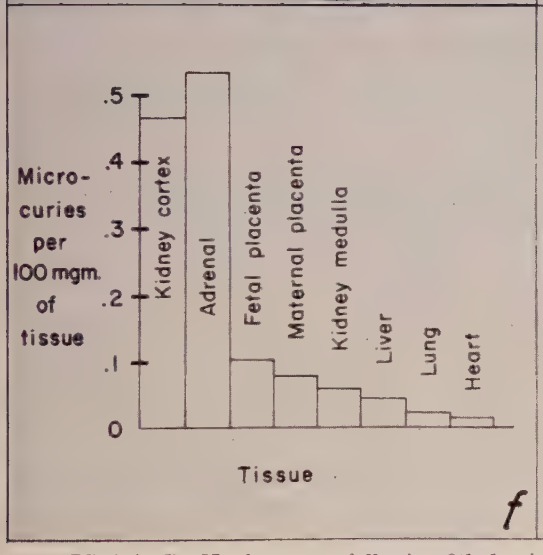
1) by producing severe disease in the mother which then indirectly affects the fetus (maternal rats exhibited marked proteinuria and weight loss following kidney antisera injections). 2) by directly interfering with embryonic growth and differentiation. 3) by interfering with placental function and permeability.

Results which helped in evaluating the possible mechanisms were that teratogenic rat kidney antiserum, when labelled with



EXPERIMENTAL TECHNIQUE: 8th day	FETUS: 21st day	
	Mortality (percent)	Weight (grams)
Operative control	8.0	4.90 ± .37
Injected rabbit plasma	15.1	4.85 ± .26
Injected rat plasma antiserum	9.2	4.74 ± .37
Injected rat kidney antiserum	36.6	4.39 ± .51

e



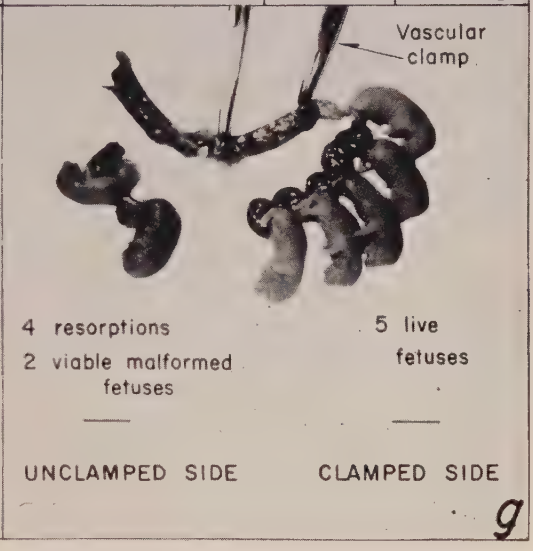


FIG. 1 (*a-d*). Newborn rats following 8th day inj. of rat kidney antiserum. *a*) Hydrocephalus, anophthalmia and absent external auditory canal. *b*) Anophthalmia, edema and evisceration. *c*) Hypoplasia of tail and evisceration. *d*) Evisceration and club feet. *e*) Control groups consisted of one-half hr laparotomy on 8th day; inj. of rabbit anti-rat plasma antisera and normal rabbit plasma on 8th day. Avg percent mortality and fetal weights in the group inj. with kidney antiserum was significantly different from control groups. *f*) Localization of I^{131} labelled kidney antiserum in rat tissues following intrav. inj. Corrected levels of I^{131} in placenta are actually much higher because of rapid placental growth in the 48 hr following inj. *g*) Clamping of one horn of uterus just before inj. of potent kidney antiserum results in a marked protective effect even when clamps are removed in one-half hr.

I^{131}, localized in maternal kidney cortex and in fetal placenta in very high concentrations and that the results of combining vascular clamping with injection of teratogenic kidney antibody showed a higher incidence of malformation and death on the unclamped side.

The fact that antibody 1) localized in fetal placental tissue and 2) that it had a significant effect in the first half hour after injection, tends to incriminate a disturbance in placental function as a major factor in production of these malformations, although neither of the other 2 postulated mechanisms can be presently eliminated. However, it is

significant that an antibody which was undetectable in maternal rat blood following intravenous administration, has proven to be so potent a teratogenic agent. From both theoretical and practical standpoints, it would be most important to understand the qualitative and quantitative mechanisms by which this biologically prepared and altered protein induces malformations.

Summary. Rabbit anti-rat kidney sera, in a prescribed dosage, injected intravenously into pregnant rats on the 8th day of gestation, resulted in severe congenital malformations in 100% of the fetuses. Larger doses caused complete fetal resorption. These antisera may produce teratogenesis by interfering with placental function.

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Biosynthesis of a Sulfobromophthalein Mercaptide with Glutathione.* (26391)

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Although glutathione mercaptides have long been suggested as intermediates in mercapturic acid synthesis(1-4), isolation and identification of a glutathione mercaptide from biological fluids has proved difficult.

The dye sulfobromophthalein (BSP) is rapidly taken up by the liver and secreted into the bile as pigmented metabolites(5-8). The main metabolite appearing in human bile has been identified as an unacetylated mercaptide formed through the –SH group of cysteine(9). The consistent demonstration of both glycine and glutamic acid in acid hydrolysates of this metabolite(5,7-9) has suggested that it may be a BSP-glutathione mercaptide(9,10). Positive identification of the glutathione mercaptide was difficult since both glycine and glutamic acid were found in acid hydrolysates of identically prepared BSP-free fractions from normal bile(5,9).

The present report describes the successful incorporation of isotopic glycine, glutamic acid and cysteine into the major BSP metabolite synthesized by the isolated perfused liver of the rat. The results indicate that BSP is excreted into bile primarily as a mercaptide with glutathione.

Methods. Intact livers were taken from Long-Evans male rats, weighing 400-450 g, and perfused(11). At 1 hr 50-125 μc of either glycine-U-C^{14}, glutamic acid-U-C^{14}, glutamine-U-C^{14}, cystine-S^{35} or glutathione-S^{35} (Schwartz Laboratories) was added to the perfusate. After 30 min equilibration, bile to be used in the controls was collected for 30 min and immediately frozen. BSP, 20 mg, was then introduced into the perfusate and equilibrated for 30 min. Experimental bile was collected during the subsequent 30 min interval. BSP levels were determined by the technic of Gaebler(12). Samples of bile or bile fractions, 10-50 μl, were plated and counted with an end-window Geiger-

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Müller counter. Duplicate samples varied less than 8% and corrections for self-absorption proved to be minimal.

Fractions of equal radioactivity from both experimental and control bile were purified identically. Samples were initially extracted with 3 volumes of acetone, and the extracts were column chromatographed as described previously(9). The first fraction obtained by employing acetone/water/concentrated NH_4OH (12.5:11.5:1 v/v) as solvent contained the bile pigments, free BSP not metabolized by the liver, some of the minor, less polar metabolites found in rat bile, and free amino acids and peptides. About 88-96% of labeled cystine, glutathione, glutamic acid, glutamine and glycine could be recovered in this fraction. The BSP metabolites were then eluted from the column, using acetone/water (1:9 v/v) as solvent. This second fraction was concentrated by rotary evaporation under reduced pressure at 40°, dried *in vacuo,* redissolved in a minimal quantity of water and chromatographed on washed Whatman 3 MM paper(9) in an ascending system employing n-butanol/water (1.48:1 v/v) made into a single phase solution with 0.27 volume of acetic acid. The metabolite fraction located by exposure to ammonia fumes was eluted with water and rechromatographed in *tert* butanol/water (1.73:1 v/v). Serial segments of each chromatogram from origin to solvent front were cut out and counted. The incorporation of label into the metabolized fraction was compared to that in the remaining portion of the chromatogram.

Both the purified metabolites and comparable control fractions were hydrolyzed in 6 N HCl at 120° for 15 hr. HCl was removed by repeated evaporation to dryness of water solutions. The labeled products were separated and their mobilities compared to those of reference amino acids on Whatman No. 1 paper employing the *tert* butanol system.

In 4 nonisotopic experiments the metabolite was isolated from the bile and serially purified as described above. Total BSP and free amino nitrogen content(13) were determined on aliquots taken before and after acid hydrolysis. The hydrolysate was chromatographed in the *tert* butanol/water system. The fractions corresponding in mobility to glycine, glutamic acid and cysteine were eluted with water and their molar relationship was determined with ninhydrin.

Results. Acetone extracts of experimental bile contained 3 chromatographically distinct forms of the secreted BSP(5). Both free BSP and a fast-moving metabolic product were removed in the initial eluate on the alumina column. Thus, the slow-moving product, which in the isolated perfused liver system represented 85-90% of the secreted BSP, was the only fraction investigated.

When either cystine-S^{35}, glutathione-S^{35}, glutamic acid-C^{14}, glutamine-C^{14}, or glycine-C^{14} was perfused through the isolated liver preparation (Table I), approximately 25-50% of the label appearing in the experimental bile was associated with the BSP metabolite fraction from the alumina column. Subsequent purification of the metabolite by 2 paper-chromatographic systems removed only small amounts of the individual label. Fractions of control bile after similar purification showed no significant isotopic activity.

The hydrolyzed BSP metabolite obtained after glycine perfusion yielded a single major radioactive spot representing 90% of the total label and corresponding in mobility to reference glycine. Similarly, hydrolysis of glutamic acid-C^{14} and glutamine-C^{14} metabolites resulted in the appearance of radioactivity in the glutamic acid position in the chromatogram. Hydrolysis and chromatography of S^{35}-containing metabolites from liver perfused with cystine or glutathione produced ill-defined S^{35}-active fractions at the origin, at the Rf of cysteine (0.15) and the degraded pigmented moiety (0.92), and at the solvent front.

The BSP and free amino nitrogen content of the purified unlabeled metabolite before and after acid hydrolysis is shown in Table II. Control bile fractions, although ninhydrin-negative before hydrolysis, contributed some ninhydrin-positive material after treatment with HCl (probably arising from impurities in the chromatographic paper). After

TABLE I. Levels of Isotopic Amino Acids and Glutathione in BSP Metabolites and Control Fractions at Successive Stages of Purification. Samples of control and experimental bile containing equal radioactivity (taken as 100%) were serially purified by column and paper chromatography. Results are reported as percentage of radioactivity in the metabolite fractions.

	Acetone/water eluate from alumina column		Paper chromatography			
			n-butanol water/HAc*		*tert* butanol/water†	
	Control	Exp.	Control	Exp.	Control	Exp.
Cystine-S^{35}	10	29	1	21	0	19
	10	27	1	23	0	22
	10	26	1	20	0	18
Glutathione-S^{35}	10	24	1	22	0	20
	9	28	0	24	0	23
Glutamic acid-C^{14}	5	52	0	49	0	47
	8	34	2	28	0	28
Glutamine-C^{14}	5	38	0	33	0	30
Glycine-C^{14}	7	60	1	21	0	17
	6	40	0	26	0	25

* Metabolite fraction: Rf = 4.0-5.0.

† Metabolite fraction: Rf = 4.0-4.5.

correction for these impurities, approximately 3 μM of amino nitrogen were found for each μM of the BSP present before hydrolysis.†

The mean molar ratio of glycine, glutamic acid and cystine, determined after chromatographic separation of the hydrolysates, was 1:0.91:0.18 (Table III). In agreement with other reports(5,7,8) no other amino acids were detected, except alanine in small amounts.

Effect of cysteine concentration on synthesis of glutathione mercaptide. To determine whether cysteine may compete with glutathione for the synthesis of BSP mercaptides, 250 mg of cysteine hydrochloride was dissolved in minimal water. After being adjusted to pH 7.4, the solution was added to the perfusate of a glycine-C^{14} experiment. (Higher concentrations of cysteine proved toxic to the perfused liver.) Neither the percentage of secreted BSP appearing as the major slow-moving fraction (91%) nor the uptake of glycine-C^{14} into the purified metabolite (23%) was affected by the added cysteine.

TABLE II. Appearance of Amino Nitrogen after Acid Hydrolysis of Purified BSP Metabolite. Unlabeled BSP metabolite purified by the 3 chromatographic procedures described in text was hydrolyzed in 6 N HCl for 20 hr at 120°. After removal of HCl from the hydrolysate, total amino nitrogen was determined by method of Moore and Stein(13).

Perfusion	Purified control fraction		Purified metabolite of BSP		Metabolite minus control		BSP in metabolite sample	N/BSP, μM/μM
	μM	N	μM	N	μM	N	μM	
1	1.5		2.6		1.1		.4	2.8
2	1.1		2.4		1.3		.4	3.3
3	1.1		5.5		4.4		1.3	3.4
4	1.2		2.2		1.0		.3	3.3

† This calculation assumes that free and conjugated BSP have the same molecular extinction coefficient. Accurate determination of the constant for the conjugate must await its availability in crystalline, pure form.

Discussion. Incorporation of glycine-C^{14}, glutamic acid-C^{14} and cysteine-S^{35} into the major BSP metabolite appearing in bile from isolated perfused rat liver and their absence from comparable control fractions strongly support the previous suggestions that the pigment is conjugated, at least in part, by the liver with glutathione(9).

Since both C^{14}-glycine and C^{14}-glutamic acid were isolated intact after hydrolytic degradation of the respective metabolites resulting from perfusion of the two amino acids, the isotopes present in the metabolite could not have resulted from degradation of the C^{14}-labeled amino acids and the ultimate entrance of their C^{14} fragments into the carbon pool and thence into the carbon skeleton of cysteine itself(14).

That the mercaptide conjugate of BSP

TABLE III. Recovery of Glycine, Glutamic Acid and Cysteine after Acid Hydrolysis of BSP Metabolite. Hydrolyzed metabolite (see Table II for details), after removal of HCl, was subjected to paper chromatography in the *tert* butanol/water system. Fractions corresponding in mobility to glycine, glutamic acid and cysteine were eluted with water and their amino nitrogen was determined. Results are given as μg N in sample chromatographed.

	Glycine		Glutamic acid		Cysteine		Ratio after subtracting control values
Perfusion	Control	Exp.	Control	Exp.	Control	Exp.	(gly : glu : cyst)
1	4.7	14.9	5.3	13.6	0	2.5	1 : .81 : .25
2	4.1	12.0	4.6	11.8	1.8	1.8	1 : .91 : .0
3	3.4	30.8	3.5	29.4	0	4.8	1 : .94 : .17
4	5.3	12.0	5.2	11.8	0	2.0	1 : .98 : .30

contains glutathione is further suggested by the observations that a) 3 M of ninhydrin-positive amino groups per M of BSP were liberated after acid hydrolysis, b) except for small amounts of alanine (presumably arising as a hydrolytic product of cysteine), only glycine, glutamic acid and cysteine were detectable, and c) glycine and glutamic acid were present in equal molar relation. (Since cysteine is extensively degraded during hydrolysis with mineral acids(9), the low recovery of this amino acid was not surprising.) Incorporation of glutamine into the glutamic acid moiety of the conjugate was expected since glutamine is rapidly transferred into liver cells where it can be hydrolyzed to glutamic acid by glutaminase(15). Glutathione-S^{35} also acted as a precursor for glutathione conjugation, although from these experiments alone it was not possible to conclude whether the glutathione was incorporated intact into the glutathione mercaptide or whether it acted solely as a source of cysteine which was then incorporated into some activated glutathione precursor.

During this investigation, Booth, Boyland and Sims(16) reported successful synthesis of a naphthalene glutathione conjugate in rat liver slices. The results of their work and our isotopic studies leave little doubt that the liver synthesizes mercaptide conjugates containing glutathione. The following scheme(17) for mercapturic acid synthesis has been suggested:

R + glutathione → R-glutathione → R-cysteine + glycine + glutamic acid → R-acetylcysteine (mercapturic acid).

Studies *in vivo* have demonstrated that enzymes are available for hydrolysis of a glutathione mercaptide to a cysteine mercaptide(4,17). The primary site of this enzymatic activity may be the kidney(16). Although small amounts of BSP metabolites have been found in urine from hepatectomized dogs(18), BSP is normally excreted directly from the liver into the bile, which may explain why it is found primarily as a glutathione conjugate.

The observation that high levels of cysteine added to the perfusate did not significantly affect the synthesis of BSP glutathione supports the concept that cysteine and glutathione do not compete directly for molecules to be conjugated but instead that mercaptide conjugation with glutathione is a specific, enzymatically controlled step preceding mercapturic acid synthesis.

Summary. The incorporation of glycine-U-C^{14}, glutamic acid-U-C^{14}, glutamine-U-C^{14}, cystine-S^{35} and glutathione-S^{35} into the major mercaptide conjugate of sulfobromophthalein (BSP) was studied in bile from isolated perfused rat liver. Incorporation of label from each of the amino acids was demonstrable in the purified metabolite obtained after 3 serial chromatographic steps, whereas control fractions were negative. Acid hydrolysis of the metabolite and subsequent paper chromatography revealed that the amino acids had been incorporated intact, that 3 M of amino nitrogen were released for each M of BSP, and that glycine and glutamic acid were in equimolar ratio (cysteine was degraded during hydrolysis and could not be quantitatively recovered). High concentrations of cysteine in the perfusate did not compete with glutathione for the synthesis of BSP mercaptide. It was concluded that BSP

is secreted into bile, at least in part, as a mercaptide conjugate with glutathione.

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Urea Derivatives as Fibrinolysis Promoting Agents.* (26392)

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During the search for synthetic compounds to enhance the induction of fibrinolysis in human plasma by urokinase, Cibalgin,® an analgesic drug, was found to be active. Subsequent testing showed that enhancement was derived from 2 components, urethan and ethylurea. A variety of other urea derivatives was then found with similar properties. Controls revealed that some of these compounds slowly dissolved plasma clots in absence of urokinase. We therefore undertook to determine whether small amounts of such non-toxic chemicals could induce activation of the fibrinolytic enzyme system *in vitro.*

Material and methods. The preformed rotating standard clots were prepared from either fibrinogen or human plasma as previously described(1), except that a rotating device was used instead of a rocking device. This arrangement permits study of fibrinolysis promoting agents acting upon one well defined invariable surface of a preformed standardized clot. The disintegration products are continuously removed from the clot surface, and concentration of the agents being tested, in contrast to fibrin plate method, does not vary to a great extent. The progress of the clot lysis is read at intervals in terms of ml of digested clot.

Human plasma clots. Human ACD bank plasma (3 to 5 weeks old) or fresh plasma (1 part Na-citrate USP 0.129 M to 4 parts blood, centrifuged 5 minutes at 1500 g) was clotted by addition of 0.05 ml $CaCl_2$ (0.5 M) per ml for standard clots or by 0.01 ml thrombin for test tube studies. There was no consistent difference in the results of experiments using fresh or stored plasma.

Bovine fibrin standard clots. Armour bovine fibrinogen (fraction I from bovine plasma) was dissolved to 0.4% in borate buffer pH 7.4 and clotted by addition of 0.01 ml thrombin per ml fibrinogen solution.

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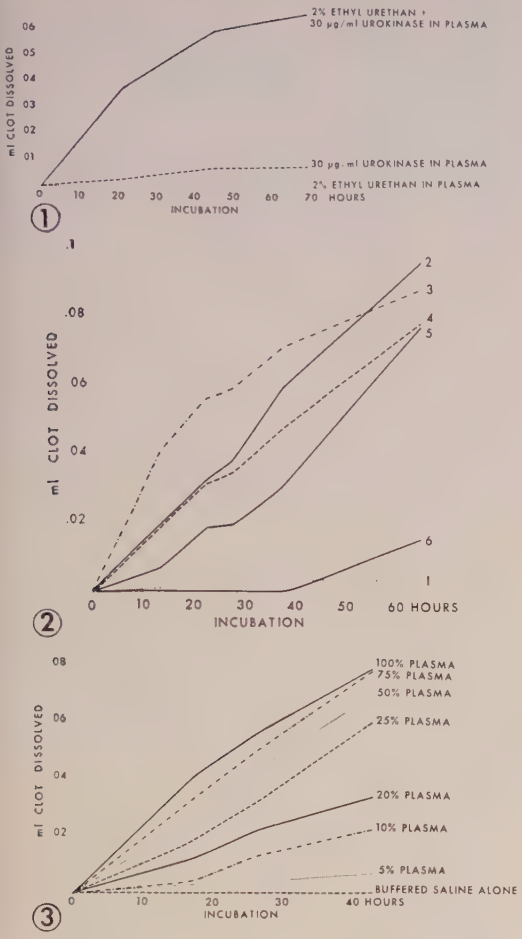

FIG. 1. Enhancement of urokinase-induced lysis of a preformed human plasma standard clot by ethyl urethan.

FIG. 2. Progressive dissolution of preformed human plasma standard clots by urea derivatives: allylurea (1), ethylthiourea (2), ethyl urethan (3), methyl urethan (4), thiourea (5), urethan (6). Note early and marked clot dissolution by ethyl urethan.

FIG. 3. Dissolution of a preformed bovine standard fibrin clot by 4% ethyl urethan in presence of increasing amounts of human defibrinated plasma. Note: Ineffectiveness of ethyl urethan in absence of human plasma and increasing activity with increasing plasma concentrations.

Bovine fibrin plates. Fibrinogen solution was prepared and clotted as above. 8 ml were used in flat bottom Petri dishes 9 cm diameter.

Defibrinated human plasma was prepared by addition of 0.01 ml thrombin per ml plasma. It was used to avoid deposition of fibrin from plasma on the surface of the standard clot, caused by thrombin diffusing out of the clot.

Urokinase was prepared as previously described(1).

Borate buffer. 0.25 M borate-saline buffer, pH 7.4(2).

Buffered saline. One part Na-barbital acetate buffer, pH 7.4, was mixed with 4 parts NaCl 0.85%.

Thrombin. Thrombin Topical Parke Davis, 200 U/ml in 50% glycerol.

Urea derivatives. These compounds, obtained from Eastman Kokak, Rochester, N. Y., or K & K Laboratories, Jamaica, N. Y., were dissolved in defibrinated human plasma or buffered saline and the solutions were adjusted when necessary to pH 7.4.

All studies were done at 37°C.

Results. 1) *Enhancement of action of urokinase on human plasma clots by urea derivatives.* Pilot studies indicated that among the urea derivatives tested, ethyl urethan best enhanced the urokinase-induced lysis of human plasma clots, followed by methyl urethan and ethylthiourea. Enhancement of urokinase-induced fibrinolysis of the preformed plasma clot by 2% ethyl urethan was clearly demonstrable (Fig. 1). The enhancement was also quite evident when plasma clots were formed in presence of 3% ethyl urethan and small amounts of urokinase. Controls, however, revealed that 3% ethyl urethan alone slowly dissolved the clot.

2) *Dissolution of human plasma clots by various urea derivatives.* Since it was observed that human citrated plasma clots formed by thrombin in presence of 3% ethyl urethan dissolved spontaneously after about 18 to 24 hours of incubation, other urea derivatives were similarly tested. Table I represents results of a characteristic experiment with urea derivatives which were effective at a concentration low enough to permit clotting of plasma by thrombin.

At the effective concentrations listed in Table I, only the plasma solution containing 2% ethyl urethan could be clotted with $CaCl_2$. This clot also dissolved as did those clotted with thrombin. Human plasma, freshly drawn with siliconized equipment and al-

TABLE I. Spontaneous Dissolution of Human Plasma Clots Formed in Presence of Various Urea Derivatives.

Compound	Conc., %	Molarity	Dissolution time, hr
Urethan	4	.449	40
Methyl urethan	3	.291	40
Ethylthiourea	3	.288	16
Butylurea	3	.258	16
Allylthiourea	3	.258	21
Ethyl urethan	2	.171	24
None	—	—	>72

lowed to clot spontaneously in glass tubes in presence of 2% ethyl urethan redissolved within 20 to 24 hours.

When preformed standard clot was used, the urea derivatives were dissolved in human defibrinated plasma at a concentration of 5%. The dissolution rates for the most active compounds are shown in Fig. 2. At this concentration, urea, methylurea, ethylurea, 1, 1-diethylurea, 1,3-diethylurea were inactive. In all experiments ethyl urethan exhibited the strongest clot-dissolving activity. It was also observed that ethyl urethan produced a slight turbidity after incubation with plasma, but clearing by centrifugation did not affect the results. The other urea derivatives produced various degrees of turbidity, including those that were inactive. Ethylthiourea, which showed good activity, produced no turbidity.

3) *Dissolution of bovine fibrin clots by ethyl urethan. Requirement of a plasma factor.* The most active compound, ethyl urethan, dissolved to 4% in buffered saline, was completely inactive on bovine fibrin plates or on standard clots formed with bovine fibrinogen. The clot-dissolving property could be restored when ethyl urethan was mixed before application with human plasma instead of buffered saline (other compounds were not tested). Fig. 3 reproduces results of a representative experiment with the bovine fibrin standard clot.

On bovine fibrin plates, the compound also became effective and produced marked lysed zones when mixed with plasma instead of buffered saline. Preincubation of ethyl urethan with plasma for about 4 hours was necessary to produce the maximal effect. Dialysis of the ethyl urethan-plasma mixture against citrated buffered saline removed the ethyl urethan, but only slightly reduced the fibrin dissolving activity of the non-dialysable material.

Discussion. It was first assumed that the plasma clot dissolution by urea derivatives was due to fibrin depolymerization alone. However, the inability of the compounds to dissolve thrombin-clotted fibrin consisting of fibrin s, soluble in 5 M urea(3), in absence of plasma, and dissolution of human plasma clots consisting of fibrin i (urea-insoluble) by some urea derivatives at concentrations much lower than 5 M, suggested that the compounds act by a different mechanism than that of urea. Juehling and Woehlisch (4) demonstrated induction of fibrinolytic activity in equine and porcine plasma incubated with urea and this plasma retained its fibrinolytic activity (demonstrated on clots obtained from pure fibrinogen) after the urea had been removed by dialysis. Identical treatment of a equine or porcine fibrinogen solution with urea did not produce the same phenomenon, so these authors specified the requirement of a plasma factor for this particular urea activity. Their critical urea concentration was 15%, whereas the critical concentrations of the urea derivatives in the experiments reported here were 2 to 4%.

The mechanism and factors involved in this chemically induced fibrinolytic activity of plasma and their relationships to *in vitro* activation of the fibrinolytic system by chloroform(5) or by acetone precipitation at acid pH(6) require further study. Retention of fibrinolytic activity after removal of an active compound by dialysis, and the necessity for presence of human plasma for dissolution of bovine fibrin indicate that a plasma factor is involved which might be related to proactivator.

To our knowledge it has not been previously reported that a non-enzymatic compound added to human plasma before clotting induced spontaneous dissolution of the clot. This experimental observation combined with use of the human standard clot may permit discovery of more active com-

pounds than those described here. Induction of fibrinolytic activity in human plasma by addition of such compounds may lead to new approaches for development of thrombolytic agents.

Summary. A number of urea derivatives added at low concentrations to plasma before or after clotting induce spontaneous dissolution of human plasma clots. They also considerably enhance the activity of urokinase. Ethyl urethan was the most active compound of the group. Bovine fibrin clots are dissolved by ethyl urethan only in presence of human plasma.

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Metabolism of 5-Hydroxyindole Compounds in Experimentally Produced Phenylketonuric Rats.*† (26393)

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The studies of Jervis(1) have shown that the primary biochemical defect in phenylketonuria consists of an inability to oxidize phenylalanine to tyrosine due to absence of the liver enzyme, phenylalanine hydroxylase. This results in an accumulation of phenylalanine in the blood and excretion of phenylketones in the urine. Armstrong and Robinson (2) and Ferari *et al.*(3) have demonstrated the presence of indolelactic acid in urine of phenylketonuric patients and suggested that there may also be a disorder of tryptophane metabolism in this disease. More recently, 3 different groups(4-6) have observed a significant lowering of 5-hydroxytryptamine in the serum and of 5-hydroxyindoleacetic acid in the urine of phenylketonuric patients. These changes are reversible when the patients are placed on a diet low in phenylalanine content. Pare *et al.*(7) have suggested that this disturbance of 5-hydroxyindole metabolism in phenylketonuria is due to an inhibition of 5-hydroxytryptophan decarboxylase by phenylalanine metabolites, a finding which has been confirmed by both *in vitro*(8) and *in vivo*(9,10) studies.

Auerbach *et al.*(11) showed that phenylketonuria could be produced experimentally in rats by feeding excessive amounts of both phenylalanine and tyrosine. This provided a means for studying the metabolism of the 5-hydroxyindole compounds in an *in vivo* system which is genetically normal.

Materials and methods. Phenylketonuria was produced experimentally according to the procedure described by Auerbach *et al.* (11). Weanling rats of the Sprague-Dawley strain were placed on stock diets and experimental diets as follows: 1) 3.75% L-tyrosine and 3.75% DL-phenylalanine for 3 months, 2) 3.75% L-tyrosine and 3.75% DL-phenylalanine for 2 months, 3) 3.75% L-tyrosine and 2.5% DL-phenylalanine for 2 months, and 4) 2.5% DL-phenylalanine alone for 2 months.

Twenty-four hour collections of urine were obtained weekly and phenylpyruvic acid was determined by the method of Berry and Woolf(12) and 5-hydroxyindoleacetic acid by the method of Udenfriend *et al.*(13). Blood samples were obtained by cardiac puncture at 6½ to 7 weeks and 9 weeks after start of the diet. The plasma was analyzed for 5-hydroxytryptamine using the method

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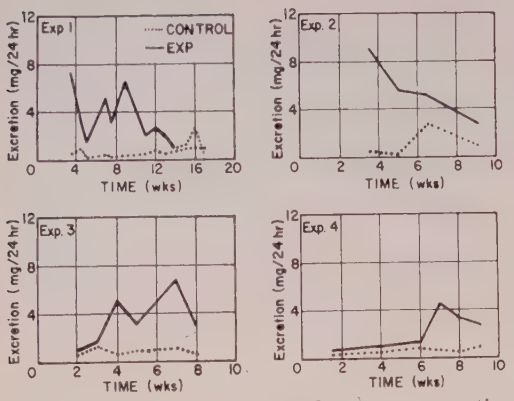

FIG. 1. Mean urinary phenylpyruvate excretion expressed as mg/24 hr for rats fed stock and experimental diets.

Exp. 1. 3.75% L-tyrosine + 3.75% DL-phenylalanine.
Exp. 2. 3.75% L-tyrosine + 3.75% DL-phenylalanine.
Exp. 3. 3.75% L-tyrosine + 2.5% DL-phenylalanine.
Exp. 4. 2.5% DL-phenylalanine.

described by Waalkes(15) and phenylalanine by the spectroscopic method of LaDu and Michael(14). Because of the high levels of tyrosine in the experimental rats, the plasma from these animals was first chromatographed with n-butanol saturated with 2N NaOH, the section of the filter paper containing tyrosine removed, and the remaining fraction eluted with distilled water and concentrated before being used for determination of phenylalanine.

Liver phenylalanine hydroxylase was determined by the method of Kenney *et al.* (16). One milliliter of soluble fraction of liver prepared from 33% homogenates in 0.15 M KCl by centrifugation in a preparatory ultracentrifuge at 10,500 g for 30 minutes was shaken at 35°C for 60 minutes with 150 μM of potassium phosphate buffer (pH 6.8) 2 μM of phenylalanine, 2 μM of DPNH, and 5 μM of nicotinamide in a total volume of 1.75 ml. At the end of incubation, 0.25 ml of water was added, and protein was precipitated by 0.5 ml of 30% trichloroacetic acid. 0.2 ml of clear supernatant was used for tyrosine determination by the colorimetric method of Udenfriend and Cooper (11). Protein content of each homogenate was determined by measuring the absorption at 260 and 280 mμ, and calculated as follows: Protein concentration (mg/ml) = 1.55 $O.D._{280}$ – 0.76 $O.D._{260}$. Amount of enzyme activity was expressed as μM of tyrosine formed per 100 mg of protein.

Results. Mean values of the 24 hour excretion of phenylpyruvate are given in Fig. 1. The majority of the experimental animals placed on both phenylalanine and tyrosine were found to excrete increased amounts of phenylpyruvate as soon as sufficient amounts of urine could be obtained for analysis. This increased to a peak level of 5 to 6 mg/24 hours at 7 to 9 weeks after being placed on the diets. Phenylpyruvate excretion reverted to control levels when phenylalanine and tysosine supplementation was discontinued. When the rats were fed 2.5% DL-phenylalanine alone, there was a delay in increase of phenylpyruvate excretion until the animals had been on the diet for 4 to 6 weeks.

Plasma phenylalanine levels obtained at 6½ weeks to 7 weeks and at 9 weeks showed a similar and statistically significant rise in experimental animals fed phenylalanine and tyrosine (Table I). However, the animals fed 2.5% DL-phenylalanine alone failed to show a significant increase.

Mean values of urinary 5-hydroxyindoleacetic acid excretion in these same animals are shown in Fig. 2. There was a decrease in experimental animals fed tyrosine and phe-

TABLE I. Blood Phenylalanine Levels Expressed as μg/ml in Rats Fed Stock and Experimental Diets.

Diets	Weeks on diet 6½ to 7	9
Control	18.6 ± 9.8 (3)	8.8 ± 6.0 (4)
3.75% L-tyrosine & 3.75% DL-phenylalanine	34.6 ± 6.2 (3)	33.1 ±13.3 (4)
Control	12.7 ± 7.1 (2)	7.3 ± 6.7 (5)
3.75% L-tyrosine & 2.5% DL-phenylalanine	42.4 ±14.0 (5)	32 ±13.9 (4)
Control	9.3 ± 4.8 (4)	—
2.5% DL-phenylalanine	10.5 ± 1.9 (3)	—

Numbers in parentheses indicate No. of animals sacrificed in each experiment. Values expressed as means and stand. dev.

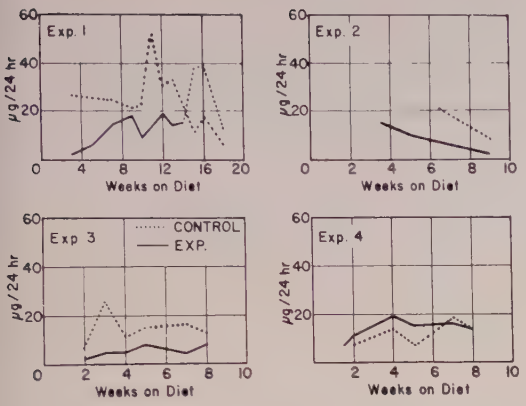

FIG. 2. Mean urinary 5-hydroxy indoleacetic acid excretions expressed as μg/24 hr for rats fed stock and experimental diets.

Exp. 1. 3.75% L-tyrosine + 3.75% DL-phenylalanine.
Exp. 2. 3.75% L-tyrosine + 3.75% DL-phenylalanine.
Exp. 3. 3.75% L-tyrosine + 2.5% DL-phenylalanine.
Exp. 4. 2.5% DL-phenylalanine.

nylalanine and a close correlation existed between rise of phenylpyruvate and fall of 5-hydroxyindoleacetic acid. No such decrease could be found in the animals given 2.5% DL-phenylalanine alone.

Plasma 5-hydroxytryptamine levels at 6½ to 7 and 9 weeks are shown in Table II. There was a significant decrease noted in the experimental animals fed phenylalanine and tyrosine. However, no such difference could be observed among the animals given 2.5% DL-phenylalanine alone.

TABLE II. Blood 5-Hydroxytryptamine Levels Expressed as μg/ml in Rats Fed Stock and Experimental Diets.

Diets	Weeks on diet 6½ to 7	9
Control	.88 ± .08 (5)	.81 ± .22 (5)
3.75% L-tyrosine & 3.75% DL-phenylalanine	.48 ± .10 (5)	.42 ± .09 (5)
Control	.86 ± .18 (10)	.83 ± .17 (10)
3.75% L-tyrosine & 2.5% DL-phenylalanine	.58 ± .12 (10)	.51 ± .15 (10)
Control	.55 ± .13 (5)	.70 ± .21 (5)
2.5% DL-phenylalanine	.57 ± .08 (5)	.66 ± .31 (5)

Numbers in parentheses indicate No. of animals sacrificed in each experiment. Values expressed as means and stand. dev.

Discussion. From these data it would appear that phenylketonuria can be experimentally produced in the rat by supplementation of both phenylalanine and tyrosine to the diet. This effect is not nearly as marked when phenylalanine alone is administered. Auerbach *et al.*(11) have suggested that simultaneous administration of tyrosine might cause an adaptive decrease of phenylalanine hydroxylase in the liver. We have repeated these studies and found enzyme activity to be decreased only slightly (Table III). This discrepancy may in part be caused by differences in the methods used for assay of phenylalanine hydroxylase in the two studies.

The present data demonstrate that the decrease of 5-hydroxyindole compounds in phenylketonuria is a secondary result of excessive phenylalanine levels, and does not represent a second genetically determined metabolic lesion. This is shown by taking a normal animal, producing a metabolic lesion resembling phenylketonuria, and getting a decrease of the 5-hydroxyindole compounds. Finally, removal of the added phenylalanine and tyrosine from the diet resulted in a reversal to normal. The possible role of phenylalanine compounds upon 5-hydroxytryptophan decarboxylase in the intact animal is currently being investigated.

The etiology of the mental defect in phenylketonuria is unknown. However, it has been shown that if such children are treated with a low phenylalanine diet at an early age, near-normal mental development will result. A similar course of treatment in an older affected child is of little or no value(18). Recently, we(19) have reported on developmental pattern of 5-hydroxytryptophan decarboxylase in the newborn rat kidney. There is an absence of the enzyme in the fetal and newborn rat, and activity increases gradually to adult levels by 33 days of age. One could speculate that the excessive amounts of phenylalanine in the phenylketonuric child would further inhibit an already immature enzyme system, and this in turn

TABLE III. Liver Phenylalanine Hydroxylase Activity Expressed as μM Tyrosine Formed/100 mg Protein in Rats Fed Stock and Experimental Diets.

Wk on diet	Controls	3.75% L-tyrosine & 2.75% DL-phenylalanine	3.75% L-tyrosine & 2.5% DL-phenylalanine	2.5% DL-phenylalanine
1	4.42 ± .94 (7)	3.25 ± .82 (7)	—	—
2	4.44 ± 1.10 (7)	3.46 ± .84 (7)	—	—
3	4.39 ± 1.30 (7)	4.34 ±1.10 (7)	—	—
4	4.73 ± 1.10 (7)	3.27 ± .41 (5)	—	—
6½ to 7	4.33 ± 1.60 (8)	4.11 ± .90 (5)	4.08 ± .27 (5)	5.31 ± 2.36 (5)
9	4.94 ± 2.15 (10)	3.48 ± .89 (6)	4.18 ± .86 (5)	5.05 ± 2.31 (5)

Numbers in parentheses indicate No. of animals sacrificed in each experiment. Values expressed as means and stand. dev.

will result in a decrease of 5-hydroxytryptamine which is essential to the developing brain.

Summary. Data have been presented confirming experimental production of phenylketonuria in rats. Supplementation of the diet with both phenylalanine and tyrosine resulted in a marked increase of phenylpyruvate excretion in urine and of phenylalanine level in plasma. Supplementation with 2.5% DL-phenylalanine alone showed a much less marked effect. The rise of phenylpyruvate coincided with a decerase of 5-hydroxyindoleacetic acid in the urine. Similarly, the increase of phenylalanine in the plasma coincided with a decrease of 5-hydroxytryptamine in the plasma. This indicates that the disturbance of 5-hydroxyindole metabolism in phenylketonuria is secondary to the increase in phenylalanine. The possible significance of these phenomena in the etiology of mental deficiency and phenylketonuria has been discussed.

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Increased Adrenaline Production Following Administration of 2-Deoxy-D-Glucose in the Rat.* (26394)

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Following the observation(1) that administration of 2-Deoxy-D-Glucose (2-DG) to the eviscerated-nephrectomized rabbit decreases the amount of glucose required to maintain a constant blood sugar level, data have been presented to support the hypothesis that 2-DG acts as a metabolic blocking agent inhibiting glucose utilization. Thus, in spite of the increased blood glucose level following 2-DG in the rat(2), dog(3) and man (4), a condition results which has been termed "cellular hypoglycemia" or cellular glucopenia(2). Since the blood sugar level can affect secretion of adrenal medullary hormones we have tested the effect of 2-DG on secretion of adrenaline and noradrenaline in the intact rat. When positive results were found, additional experiments were carried out in rats with denervated adrenal glands.

Material and methods. 2-DG was obtained from the Aldrich Chemical Co., Milwaukee, Wis., through the courtesy of the Cancer Chemotherapy National Service Center, NIH, Bethesda. In all instances 2-DG was administered subcutaneously (s.c.) in water solution (50 mg/ml). In control animals a corresponding quantity of distilled water was injected. Regular insulin (Insulin Vitrum) was injected undiluted s.c.

Blood glucose concentrations were determined colorimetrically using condensation with O-toluidine(5); in this procedure 2-DG gives only one-tenth of the color produced by equal quantities of glucose within the range of 50-1000 mg %. Tail vein blood was used.

Adrenaline and noradrenaline production were judged by urinary excretion of free catechol amines, estimated fluorimetrically(6). To obtain sufficient amounts, urine was collected over 8-12 hours from groups of 2-4 rats in metabolic cages. At the end of each experiment the catecholamine content of the adrenals was estimated by the same procedure after extraction with 3% trichloracetic acid but without absorption on alumina.

The adrenal glands were denervated either by spinal cord transsection at the level of 0:7, or by division of the splanchnic nerves immediately below the diaphragm *via* the abdominal approach. Intraperitoneal barbiturate anaesthesia of short duration (Citodon-Na, Leo) was used. The rats were killed by decapitation.

Inbred male rats, 175-275 g, maintained on a diet of known composition, were used. During the acute experiment no food was given, but free access to water was allowed.

Results. It was clearly shown that 2-DG, when administered in sufficiently high doses to maintain an elevated blood sugar level for a longer period of time, produces a marked increase in adrenaline secretion. Thus, when 2-DG was given in a dose of 50 mg per 100 g body weight twice over an 11 hour period, urinary adrenaline increased about 40 times (Table I); urinary noradrenaline excretion was doubled. Secretion of adrenal medullary hormones apparently was intense enough to make resynthesis lag behind, as revealed by pronounced lowering of adrenaline content of the adrenal glands. The changes in urinary and adrenal catecholamines after 2-DG closely resembled those obtained after insulin-induced hypoglycemia over the same period (Table I). Following administration of 2-DG in smaller dosage, both the increase in catecholamine secretion and the hyperglycemic response were less pronounced.

After adrenal denervation by transsection of the spinal cord, urinary adrenaline remained normal, but a minor decrease in noradrenaline excretion probably occurred. In these rats, urinary catecholamine excretion

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TABLE I. Adrenaline and Noradrenaline Content of Adrenals and Urine after Subcutaneous Administration of 2-DG and Insulin in Intact and Spinal Rat.

	Adrenals			Urine		
	No. of rats†	Noradrenaline, μg/kg b.w.	Adrenaline, μg/kg b.w.	No. of urine samples‡	Noradrenaline, ng*/kg/hr	Adrenaline, ng*/kg/hr
Normal controls	10	17.2 ± 1.82‖	149 ± 6.7	5	73 ± 8.4	21 ± 5.1
2-DG						
25 mg × 1	4	7.8 ± .95	75 ± 10.8	1	118	153
50 mg × 1	4	11.0 ± 2.35	79 ± 21.0	1	111	367
50 mg × 2§	8	13.1 ± 2.39	32.3 ± 3.69	4	146 ± 19.1	806 ± 7.0
Insulin, 1 unit × 2§	6	15.7 ± 1.99	25.5 ± 7.32	4	197 ± 40.6	912 ± 90.3
Spinal controls	6	46.5 ± 3.95	188 ± 8.55	2	47–32	24–53
Spinal + 2-DG, 50 mg × 2§	7	43.4 ± 5.47	173 ± 9.70	2	33–42	22–53

* ng = .001 μg. † Both adrenals analyzed together. ‡ Urine from groups of 2-4 rats.
§ 5 hr interval between injections. ‖ Stand. error.

after injection of 2-DG did not change; blood glucose level probably increased (141 mg % ± 6.6, spinal control 103 mg % 2.9), although rather moderately as compared with the intact animal (264 mg% ± 13.6). Furthermore, 2-DG caused no change in adrenaline and noradrenaline content of the adrenals in the spinal rat; however, noradrenaline content was higher in the adrenals of the spinal controls as compared to normal rats. Thus, the effect of 2-DG on production of adrenaline and noradrenaline is mediated *via* a central nervous mechanism.

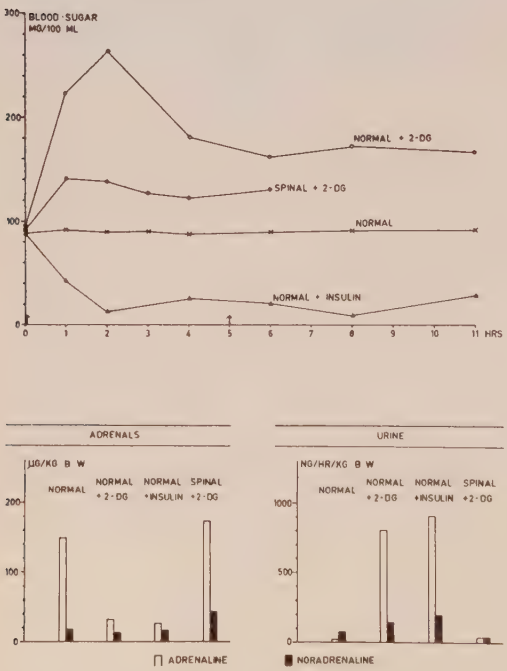

FIG. 1. Upper part: Blood sugar response evoked by 2-DG (50 mg/100 g body wt × 2) in intact and spinal rats as compared to untreated controls and normal rats given insulin (1 unit/100 g body wt × 2); ↑ denotes time of inj. (s.c.). Lower part: Adrenaline and noradrenaline in the adrenals and the urine from the same rats.

After unilateral transsection of the splanchnic nerves, observations similar to those in the spinal rats were made. Thus, after injection of 2-DG (50 mg per kg body weight × 2) the denervated adrenals contained normal amounts of adrenaline (155-156 μg per kg b.w.) while a marked decrease (to 27-32 μg) took place in the intact adrenal; the corresponding figures for noradrenaline were 45-55 μg and 27-33 μg, respectively.

Discussion. Although the symptoms appearing in animal and man after administration of 2-DG were noted by Landau *et al.*(2, 4) to be similar to those seen after insulin-induced hypoglycemia, he suggested that these phenomena were not related to adrenaline release(4). Working with anaesthetized rats Brown and Bachrach, however, could demonstrate that the hyperglycemic response to 2-DG in the intact animal was abolished by adrenal demedullation, and suggested that 2-DG is capable of stimulating adrenaline release(7). In the present studies, direct evidence supports this hypothesis. Adrenaline release of the order of magnitude seen after 2-DG has previously been found only after

convulsive doses of insulin in the rat, leading to almost total depletion of the adrenaline stored in the adrenal medulla(8). This has been confirmed in the present study. In comparison, such factors as work and hypoxia seem to cause only limited adrenaline release(8).

According to our findings, the adrenaline secretion evoked by 2-DG is the result of a central nervous stimulation. The presence of a centrally located receptor mechanism regulating adrenaline secretion was postulated in 1924 by Cannon(9) and Houssay (10), who demonstrated that the adrenaline release in hypoglycemic animals could be abolished by adrenal denervation. Dunér (11) in experiments in the cat gave some evidence that this center is located in the hypothalamus. He injected glucose locally in this area and found an inhibition of adrenaline secretion, which made him postulate a receptor mechanism sensitive to glucose. In the present studies, adrenaline release was evoked under conditions of hyperglycemia (after 2-DG) and hypoglycemia (after insulin). Blood glucose level *per se* is not the regulating factor. It seems likely that adrenaline release is linked to inhibition of glucose utilization caused by 2-DG(12). The hyperglycemia after 2-DG to a great extent depends on the release of adrenaline. That some increase in blood glucose level was still seen following 2-DG in rats after denervation of the adrenals might indicate that other factors are also involved. These studies will be enlarged, particularly in view of the report(7) that no change occurred in blood sugar level of adrenal demedullated rats after 2-DG. A pertinent question is whether 2-DG could lead to erroneously high values by interference with our procedure for blood glucose. This is unlikely, since a 2-DG level of 400 mg % would have been required to simulate an increase in blood glucose of 40 mg % as observed in spinal rats given 50 mg 2-DG per kg b.w. Further more, Landau and Lubs (2) found blood 2-DC values around 3 mg % 2 hours after s.c. administration of 40 mg of 2-DG per 100 g b.w. in the rat.

Summary. 2-deoxyglucose (2-DG) causes marked release of adrenaline from the adrenal medulla in the intact rat but not after adrenal denervation. The adrenaline released is to a great extent responsible for the hyperglycemia appearing after 2-DG, but other factors might also be involved. The inability of 2-DG to cause increased adrenaline secretion after denervation of the adrenal glands points to its action *via* a centrally located receptor mechanism regulating adrenaline secretion; this center seems not to be sensitive to the blood glucose level as such.

The authors are indebted to Drs. D. J. Ingle and M. B. Lipsett for valuable discussion and criticism.

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A New Method for Portal Venography: Retrograde Hepatic Flushing.* (26395)

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Portal venographic studies have shown characteristic changes demonstrable in a variety of disease states and have been of great value in preoperative evaluation of patients undergoing portal surgery. All current methods of portal venography in the intact patient require either needle puncture of the spleen or liver, or laparotomy and exposure of branches of the portal system. Many authors, among them Cooper *et al.*(1), have cautioned that splenic injections are not without danger, and that they would hesitate to perform the procedure on any patient who could not tolerate splenectomy.

Suggestion of a new method of portal venography. It was proposed in this study to modify the methods of hepatic venography to enable visualization of the portal venous system. The first report on hepatic venography concerned work done in dogs in which 20 ml of dye was injected into hepatic veins(2). A balloon tip catheter was used, and it was reported that the entire hepatic venous system was filled through anastomotic channels, following injection into occluded right hepatic veins. Study of injection-corrosion casts of normal dog livers failed to reveal any such anastomotic channels. Comparison of the casts of dog livers (Fig. 1) and of known portal venograms in dogs with the photograph presented in the report suggested that the venous system demonstrated was not that of the hepatic veins but was that of the portal veins. It was thought that perhaps the force of injection of 20 ml of dye into an occluded hepatic branch was sufficient to overcome the intrasinusoidal pressure and thus resulted in filling of the portal venous system in a retrograde manner. A review of the later literature on hepatic venography revealed that Celis *et al.*(3) could not demonstrate the anastomotic channels previously reported. They did find filling of vessels, however, which were postulated to be small branches of the hepatic artery or portal vein filled against blood flow. Servello(4) noted briefly that among the "snags to be avoided" in hepatic venography was placement of the catheter too far peripherally, which resulted in filling of portal branches.

Method. Ten adult, mongrel dogs (10-25 kg) were used in this study. A No. 9 Cournand catheter was introduced into the exposed external jugular vein of an anesthetized dog (sodium ethyl barbituate (Nembutal); 30 mg/kg) and was guided into the hepatic veins under fluoroscopic control. The catheter was wedged into position peripherally and then pulled free about one cm. The position of the catheter was confirmed in some cases by a check of the free and wedged pressures. A rapid injection of 20 ml of 50% diatrizoate (Hypaque) was made. Serial x-rays were obtained using an automatic cassette changer and time of exposure was noted on the films by use of an automatic timer.

In certain cases portal venograms were obtained by operative exposure and cannulation of branches of the splenic veins, or by direct puncture of the portal vein. The latter procedure was possible in dogs in which a modification of the London cannula had been placed around the portal vein.

Dogs were sacrificed at intervals of less than one hour to 12 days after the injections. Autopsy was performed and sections of the liver obtained for histologic study. Injection-corrosion casts were made by the method of Hales *et al.*(5) in some cases.

Results. Study of a series of casts of the dog liver showed that the right lateral lobes received the first branch of the main portal

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† Submitted in partial fulfillment of the thesis requirement for degree of Doctor of Medicine, Yale Univ. School of Medicine.

FIG. 1. Cast of normal dog liver, portal and hepatic veins. Note modified London cannula in position on main portal vein and the branching pattern of the portal system. (#34)

vein. It was thought that retrograde injections of these lobes would cause the dye to flow into the main portal system and then be carried to the rest of the liver. Successful catheterization of the hepatic veins was accomplished in every case. It was noted that it was easier to gain access to the right sided hepatic veins, and that as predicted, the venograms obtained when the right lateral lobes were injected did fill the whole of the portal system. Retrograde injections through the veins of the left side of the liver resulted in less filling of the portal system. Serial films showed an initial phase of hepatic venous filling (Fig. 2a) followed by a phase of sinusoidal filling (Fig. 2b) and finally a phase of retrograde filling of the portal system of the rest of the liver (Fig. 2c). The entire portal system of the liver was well visualized save the portion of the lobe injected in which the sinusoidal filling obscured the portal channels. The dye was rapidly flushed away from site of injection and the rest of the portal system.

The portal venograms obtained by splenic vein catheterization and by direct portal vein puncture confirmed that the vessels demonstrated were indeed portal veins. Additional confirmation was obtained by comparison of the films with the injection-corrosion casts (Fig. 3).

The effects of this procedure upon the liver were limited to small wedge-shaped regions at site of injection. Acutely, on occasion, small paravenous hemorrhages were noted, followed by leucocytic infiltration, coagulation necrosis, focal atrophy, and finally fibrosis. Total volume of liver tissue involved was considered to be equivalent to that lost

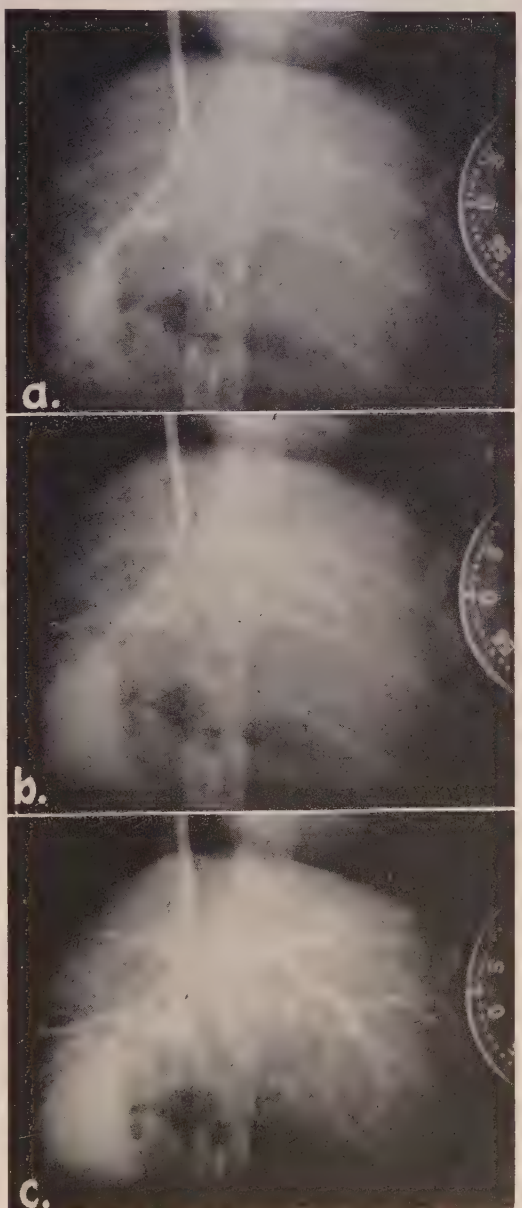

FIG. 2 a, b, c. Serial x-rays; note filling of sinusoids at catheter tip followed by progressive filling of the portal venous bed. (#25)

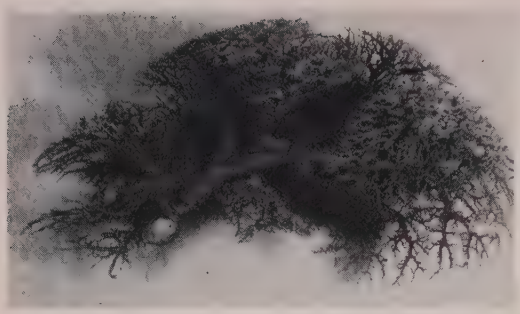

FIG. 3. Cast of portal system. (#25)

following needle puncture of the liver, with its attendant local hemorrhage. Some specimens showed no detectable changes at all.

Discussion. The proposed method for portal venography was easily performed and yielded excellent results. There was clear visualization of the entire portal system within the liver save a small section obscured by the sinusoidal filling at site of injection. The effects of the procedure upon the dog liver were studied both acutely and at intervals up to 12 days after the injections and showed minimal focal changes attributable to the procedure. Investigations are in progress in attempt to use this method of study in human patients.

Summary. A new method for portal venography by retrograde hepatic vein flushing was described. Application for the study of human cases is in progress.

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Susceptibility of Primary Cultures of Feline Renal Cells to Selected Viruses. (26396)

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Several workers have reported on the susceptibility of feline renal cell cultures to feline viruses(1-4). ECHO 10, ECMO(5), and herpes simplex(6) viruses have also been shown to be cytopathogenic in such cultures. Other human and bovine enteroviruses have been reported to be noninfective for feline renal cells(5). Since cultures of feline renal cells are a major cell type in this laboratory, their viral spectrum warranted an investigation. Twenty-four animal and human viruses were investigated for their ability to produce a cytopathogenic effect (CPE) with subsequent multiplication in primary cultures of feline renal cells. This paper reports our results.

Materials and methods. Primary cultures of feline renal cells were prepared by the trypsin digestion method described by Madin *et al.*(7). The growth medium was 0.5% lactalbumin hydrolysate in modified Hank's balanced salt solution with an addition of 10% lamb serum. The medium used at time of inoculation was lactalbumin hydrolysate containing 2% lamb serum. All media contained 500 units of penicillin and 500 μg of streptomycin per milliliter. Each of 4 renal culture tubes was inoculated with 0.1 ml of undiluted virus. When 70 to 80% of the cells were showing evidence of degeneration, 0.1 ml of fluid and cells was passed. In the absence of CPE, 0.1 ml of fluid and cells was transferred at 5- to 10-day intervals to new cell cultures. In all instances when CPE was absent the fluid was tested for virus by inoculation to susceptible cell cultures following 2 or more blind passages. No special attempts were made to adapt these viruses to

TABLE I. Susceptibility of Feline Renal Cell Cultures to Virus Infection.

Virus	Source	Primary inoculations CPE	Primary inoculations Days	Succeeding passage No.	Succeeding passage CPE	Succeeding passage Titer*
GROUP 1†						
Vesicular stomatitis (Ind.)	Allantoic fluid	+	1	10	+	4.3
" " (N.J.)	" "	+	1	10	+	3.5
Infectious bovine rhinotracheitis	Bovine kidney	+	2	10	+	6.5
Equine abortion	Hamster liver	+	4	11	+	5.5
ECHO 10	Monkey kidney	+	4	6	+	5.0
Vaccinia	FL cells	+	1	10	+	4.6
Herpes	Herpetic vesicle	+	1	10	+	6.5
Sendai	Allantoic fluid	+	1	12	+	5.0
Adenovirus 4	KB cells	+	2	7	+	3.0
GROUP 2‡						
Parainfluenza III (SF-4)	Bovine kidney	—		3 blind passages		
Canine hepatitis	Dog "	+	2	Lost at 3		
Coxsackie B-1 through B-5	KB cells	+	1	" " 3 to 5		
Adenovirus 3	HeLa cells	+	2	" " 2		
" 7	KB cells	+	4	" " 2		
GROUP 3§						
Newcastle disease	Allantoic fluid	—		3 blind passages		
Poliovirus types I, II, III	Monkey kidney	—		3 " "		
Coxsackie A-9	KB cells	—		2 " "		
ECHO 4	Monkey kidney	—		2 " "		

* Log $TCID_{50}$/0.1 ml of inoculum in feline renal cells.
† Group 1—Viruses that propagated readily, with CPE.
‡ Group 2—Viruses which produced CPE for 1 or more passages without multiplication.
§ Group 3—Viruses which did not elicit CPE nor multiply.

this tissue culture system.

*Viruses.** The viruses studied are listed in Table I.

Virus titration. Serial 10-fold dilutions of tissue culture fluid containing the virus were prepared, using nutrient fluid as the diluent. One-tenth milliliter of each dilution was inoculated into each of 4 feline renal culture tubes. The end point was calculated by the Reed and Muench method and expressed as the 50% tissue culture infective dose ($TCID_{50}$) per 0.1 ml of inoculum.

The viruses which induced a cytopathic change with subsequent multiplication were each identified following 6 or more passages by either the complement fixation, hemagglutination inhibition, or serum neutralization test.

Coverslip preparations were stained with hematoxylin and eosin following fixation in Bouin's fluid.

Results are presented in Table I. The viruses were placed in 3 groups, based upon response elicited in the cell cultures. The first of these includes the viruses that propagated readily with a cytopathogenic effect. Viruses in this group are infectious bovine rhinotracheitis (IBR), vesicular stomatitis (VS) (New Jersey and Indiana), equine abortion (EAV), vaccinia, herpes, Sendai, ECHO 10, and adenovirus type 4. Group 2 includes those viruses which produced a cytopathogenic effect for 1 or more passes but did not multiply. These are canine hepatitis, Coxsackie B-1 through B-5, parainfluenza III (SF-4), and adeno 3 and 7. Newcastle dis-

* The authors thank the following for supplying viruses for the study: W. J. Cheatham, equine abortion virus (Kentucky A); C. N. Dale, vesicular stomatitis; R. C. Reisinger, parainfluenza III (SF-4); L. R. Soma, canine hepatitis; M. D. Hoggan, vaccinia; and Dr. R. Byrnes for supplying equine abortion immune serum. The authors also acknowledge the technical assistance of Edward W. Despeaux, Armed Forces Inst. of Pathology, Washington, D.C.

Major Crandell is now at 3790th Epidemiological Laboratory, Lackland AFB, San Antonio, Texas.

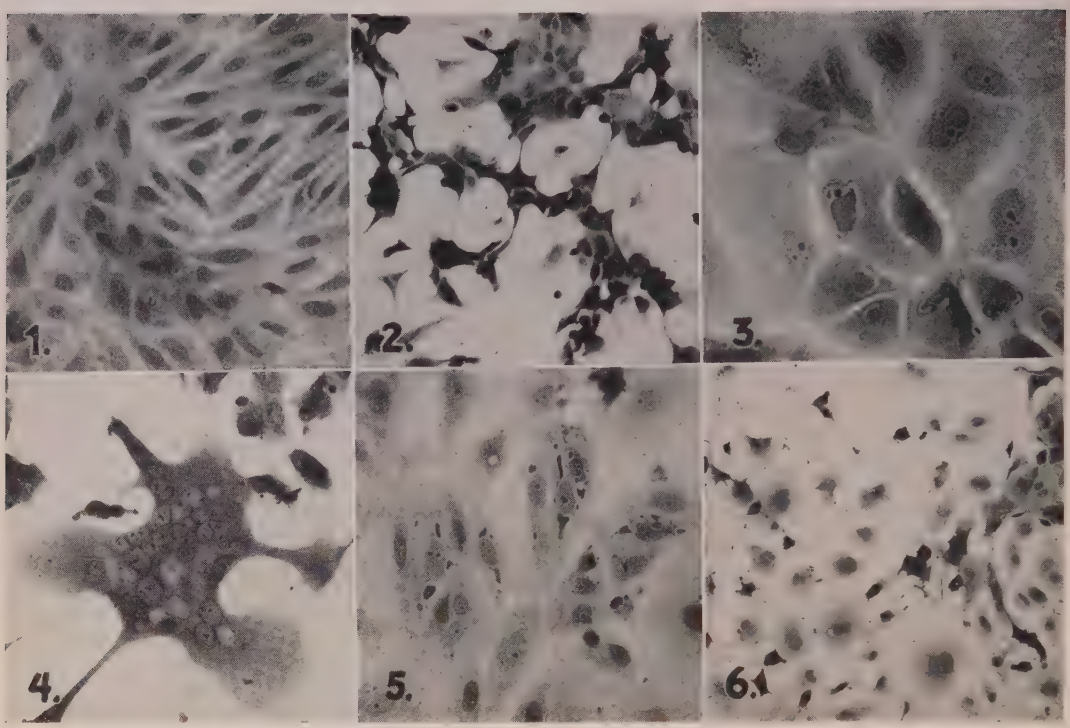

FIG. 1. Uninoculated feline renal cell culture. H & E, ×177. AFIP Neg. No. 60-3481.

FIG. 2. Cytopathic change in culture of feline renal cells infected with virus of infectious bovine rhinotracheitis. 48 hr. H & E, ×67. AFIP Neg. No. 60-3485.

FIG. 3. Intranuclear inclusion bodies in culture of feline renal cells infected with equine abortion virus. 72 hr. H & E, ×267. AFIP Neg. No. 60-3487.

FIG. 4. Syncytium in culture of feline renal cells infected with vaccinia virus. 48 hr. H & E, ×167. AFIP Neg. No. 60-3486.

FIG. 5. Cytoplasmic inclusion bodies in cultures of feline renal cells infected with adeno 4. 60 hr. H & E, ×177. AFIP Neg. No. 60-3482.

FIG. 6. Cytopathic change in culture of feline renal cells infected with Sendai virus. H & E, ×67. AFIP Neg. No. 60-3484.

ease virus (NDV), polioviruses I, II, and III, ECHO 4, and Coxsackie A-9 did not elicit a cytopathogenic change nor multiply; these comprise Group 3.

Fig. 1 shows a control culture of feline renal cells.

The cytopathogenicity with the viruses of IBR (Fig. 2), vaccinia, Sendai, equine abortion, adeno 4, VS (Ind. and N.J.), ECHO 10, and herpes simplex was very distinct. In stained preparations intranuclear inclusion bodies were demonstrated in cultures infected with IBR, EAV (Fig. 3), and herpes simplex. The cytopathogenicity of herpes simplex has been previously described in feline renal cells(6), but this is the first report, to our knowledge, of a primary isolation of this virus in these cells.

Formation of syncytia was a prominent feature in the early passages of vaccinia (Fig. 4). The cytopathic effects of VS (Ind. and N.J.), and ECHO 10 were characteristically rounding and shrinking of the cells. The presence of cytoplasmic inclusion bodies in cells infected with adeno 4 (Fig. 5) was in sharp contrast to the intranuclear inclusion bodies observed in feline cells infected with adeno 3 and 7. Cytopathogenic changes in the cultures induced by the Sendai virus were more diffusely spread throughout the culture than with the other viruses in Group 1. The cells were stellate in shape, with numerous protoplasmic bands joining adjacent cells (Fig. 6).

With all the viruses in Group 1, the titers of the fluids from the final passage indicated that the viruses had multiplied. With the exception of the VS viruses, the titers ob-

tained in feline renal cells appeared to be constant between the 5th and 10th passage for those tested. Both strains of VS virus multiplied initially with a titer of 10^{-5} but decreased considerably between the 5th and 20th serial passage.

Although the viruses in Group 2 elicited an early CPE in the cultures, they failed to multiply. During the early passages, intranuclear inclusion bodies were demonstrated in cultures infected with the viruses of canine hepatitis and with adeno 3 and 7. This behavior of these 3 viruses is another similarity between canine hepatitis virus and the adenovirus group. Although parainfluenza III (SF-4) virus did not elicit a demonstrable cytopathogenic change in the unstained cultures, both cytoplasmic and intranuclear inclusions were observed in stained preparations on the first passage.

The viruses in Group 3 did not produce a cytopathogenic effect in the feline renal cells, and infective virus could not be demonstrated when the fluid was transferred to susceptible cell cultures.

Our results with the human enteroviruses agree with previously reported data(5), except that cytopathic changes were present in early passages with Coxsackie B-1 through B-5.

Summary. The susceptibility of primary feline renal cell cultures to 24 viruses is reported. Of these, infectious bovine rhinotracheitis, vesicular stomatitis, equine abortion, vaccinia, herpes, Sendai, ECHO 10, and adenovirus 4 were shown to produce marked cytopathic effects with multiplication. Canine hepatitis virus, Coxsackie B-1 through B-5, adeno 3 and 7, and parainfluenza III (SF-4) were cytopathogenic for one or more passages but did not multiply. The polioviruses, Newcastle disease, ECHO 4, and Coxsackie A-9 had no cytopathogenic effect on the cell, and there was no evidence of viral multiplication.

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Acid Hematein Staining of Uterine Neoplastic Tissues.* (26397)

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A cytochemical method for staining phospholipids with acid hematein has been developed(1,2). Using this method to examine normal and neoplastic tissues of mice, rats, and human beings, Hori noted that non-neoplastic cells generally contain acid-hematein-positive substance in varying amounts, whereas solid tumors do not(3,4,5). The reaction also becomes less intensive in liver cells of rats during azo dye carcinogenesis (6) and of mice bearing transplanted tumors (7). The present paper deals with the reaction manifested by non-neoplastic and neoplastic cells of the human uterus.

Materials and methods. Uterine tissue specimens were obtained from 18 patients by the conventional biopsy method. They included 2 non-neoplastic cases, 12 squamous cell carcinomas, and 4 myomas. After being fixed with a formol-calcium or formol-calcium-cadmium solution for 6 hours, a portion of each tissue specimen was subjected to the acid hematein test as described in Hori's re-

* Contribution No. 468, Faculty of Science.

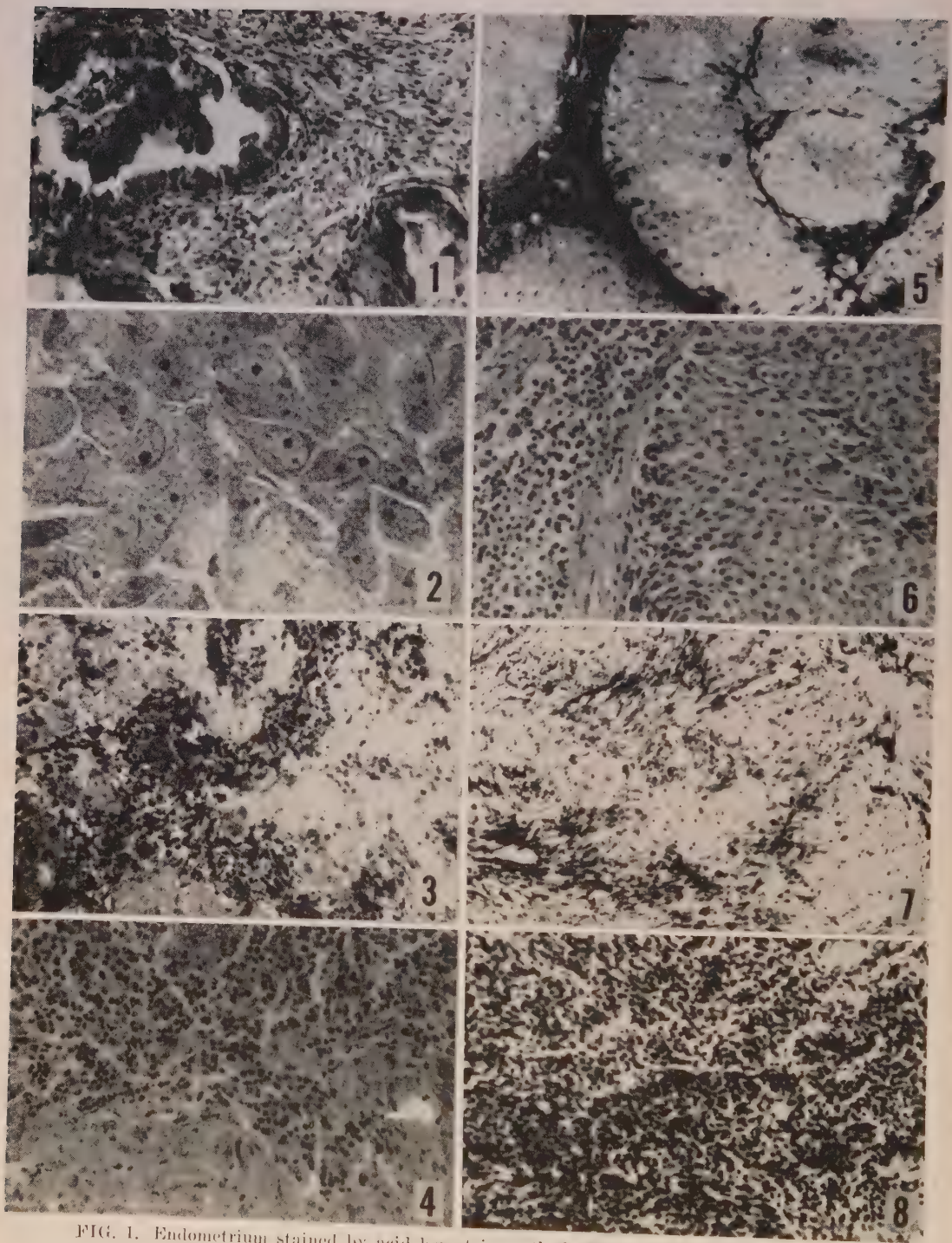

FIG. 1. Endometrium stained by acid hematein method. Note definitely positive result in epithelial cells (×150).

FIG. 2, 3, 5, 7. Uterine epithelioma stained by the acid hematein method. Note negative reaction in neoplastic cells. (Fig. 2, ×700; Fig. 3, 5, 7, ×150.)

FIG. 4, 6, 8. Hematoxylin-eosin preparations made from the same specimens used for those of Fig. 3, 5, and 7, showing tissue structures for comparison (×150).

ports(3), while the other portion was used for hematoxylin-eosin (HE) staining.

Results. Histological examinations of 18 HE preparations were carried out to confirm diagnosis. Two non-neoplastic specimens were found to be the myometrium and mucous membrane, demonstrating no atypical elements. Twelve specimens were epitheliomas, including 2 undifferentiated ones and 10 relatively differentiated ones. The remaining 4 cases were myomas.

Of the acid hematein preparations, the non-neoplastic portions showed dark blue or blue-black staining of cytoplasmic substance and nucleoplasm in the epithelial cells (Fig. 1). In some, many fine, deep blue granules were recognized lying at the juxtanuclear area in the cytoplasm. The stromal cells, the nuclei especially, were stained intensively. The myometrium was negative in reaction.

The parenchymal cells of all the epitheliomas were negative in reaction (Fig. 2, 3, 5, 7). The cytoplasm appeared pale yellow; often only the nucleoli were stained (Fig. 2). The stromal cells, however, showed dark staining, especially in the nucleoplasm (Fig. 3, 5, 7). It was often observed that the non-neoplastic epithelial cells in the adjoining areas of the epitheliomas were stained deeply. The myoma cells in all 4 cases were negative in reaction, like the muscle cells of the myometrium.

Discussion. It appears that epithelial cells and neoplastic cells of epithelial origin react differently to acid hematein lipoprotein staining, the former being definitely positive and the latter negative. It is interesting that even the non-neoplastic epithelial cells near the epithelioma are sometimes negative in reaction. Further studies with many more cases are needed to confirm the regularity of these findings. However, this particular staining method may serve to supplement prevailing conventional cytological and histological methods in biopsy as well as smear diagnosis of uterine epitheliomas.

Summary. The acid hematein lipoprotein staining was used on non-neoplastic and neoplastic human uterine biopsy specimens. Epithelioma cells are conspicuously unstained, while epithelial cells and other tissue cells show a positive reaction.

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Fatty Acid Composition of Mouse Lipids and Lipoproteins.* (26398)

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Recent advances in gas chromatographic technics have made possible the determination of the fatty acid composition of small quantities of biological lipids. The sensitivity of these technics allows measurement not only of whole plasma fatty acids, but of

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fatty acids present in the various plasma lipid esters as well as in isolated lipoprotein fractions. Thus, Dole *et al.*(1) have reported on the plasma lipid fatty acid composition for man and a number of animal species. Lindgren *et al.*(2) have determined fatty acid distribution in lipid esters of the major human serum lipoprotein fractions. The wide use of mice in biological experimentation both in this and other laboratories has prompted the present report on the fatty acid composition of plasma lipids in normal untreated mice. Also included is a comparison of mouse plasma fatty acid composition data with those obtained for humans.

Methods. Samples of plasma were obtained from 31 ten-week-old male Swiss white mice, weighing approximately 26 g, and existing exclusively on Simonsen Laboratory white diet and water *ad libitum*. Blood was drawn by heart puncture and the separated plasma was used in subsequent lipid analyses. Plasma lipoprotein fractions were isolated by the method of successive preparative ultracentrifugation previously reported(3). Three centrifugal fractions were obtained: (1) all low density lipoproteins having a density less than 1.063 g/ml, (2) high density lipoproteins (HDL) ranging in density between 1.063 to 1.218 g/ml, and (3) a sedimenting protein fraction of density greater than 1.218 g/ml. The latter fraction contains the major part of the plasma non-esterified fatty acids (NEFA) which are bound to albumin. Lipid extraction was accomplished by a modified method of Sperry *et al.*(4). NEFA extraction was carried out by the procedure of Dole(5). The extracted lipids were separated by silicic acid chromatography(6) into 3 chemical fractions: (1) cholesteryl esters, (2) glycerides, cholesterol, and NEFA, and (3) phospholipids. The amounts of lipid in these fractions were determined by infrared spectroscopy. Lipid fractions were transmethylated according to the procedure of Stoffel *et al.*(7). Methyl esters were subjected to gas-liquid chromatography using the apparatus and procedures described by Upham *et al.*(8). A 52 inch glass column (6 mm inner diameter) was packed with 48-65 mesh Chromosorb, coated with 30% by weight succinic acid diethylene glycol polyester (LAC-2R-728).† The resulting chromatograms were evaluated by a punched card technic reported by Tandy *et al.*(9). The major fatty acids are reported according to the nomenclature proposed by Dole *et al.*(1). Minor and unidentified constituents, amounting to approximately 15% of the total methyl ester weight, are designated as in a previous report(2) by: Pre 16:0, 16:1-18:0, 18:2-20:4, and Post 20:4. These designations refer to the elution position of such esters relative to the more abundant identified esters on the succinic acid diethylene glycol polyester column coating.

TABLE I. Fatty Acid Composition of Mouse Plasma Lipids. Values expressed as percentage of total fatty acid methyl esters.

Fatty acid	Cholesteryl esters	Glycerides + NEFA	Phospholipids
Pre 16:0	.4	.8	.4
Palmitic 16:0	5.0	24.8	29.9
Palmitoleic 16:1	1.5	2.8	.5
16:1 - 18:0	.1	.8	.9
Stearic 18:0	1.2	3.1	17.7
Oleic 18:1	6.0	27.2	8.2
Linoleic 18:2	50.1	33.4	30.0
18:2 - 20:4	3.4	4.1	3.7
Arachidonic 20:4	29.9	2.4	8.3
Post 20:4	2.4	.7	.6

Results. The fatty acid composition of the individual lipid compounds in mouse plasma are presented in Table I. These values show significant differences in fatty acid composition. The cholesteryl esters contain a high percentage of polyunsaturated acids, linoleic and arachidonic, with all other acids markedly low. The fraction containing both glycerides and NEFA shows palmitic, oleic, and linoleic as the principal acids. In the phospholipids, palmitic, stearic, and linoleic predominate while the lower oleic content is comparable to that of arachidonic acid.

Comparison of the mouse data can be made with normal human lipid fatty acid values (2) shown in Table II. The percentage of arachidonic acid in mouse cholesteryl esters is significantly higher than in the human. On the other hand the human cholesteryl esters have a higher percentage of palmitic and oleic acids than mouse esters. In the glyceride and NEFA fraction of the mouse the per-

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TABLE II. Fatty Acid Composition of Human Serum Lipids. Values expressed as percentage of total fatty acid methyl esters.

Fatty acid	Cholesteryl esters	Glycerides + NEFA	Phospho-lipids
Pre 16:0	3.0	3.5	2.0
Palmitic 16:0	10.0	29.8	33.2
Palmitoleic 16:1	3.2	3.7	1.1
16:1 - 18:0	1.1	1.1	.9
Stearic 18:0	1.2	4.6	14.3
Oleic 18:1	17.8	39.1	11.9
Linoleic 18:2	55.3	15.7	21.9
18:2 - 20:4	2.1	1.2	3.3
Arachidonic 20:4	5.6	1.3	9.3
Post 20:4	.6	—	2.0

centage of linoleic acid is approximately double that in the human. However, oleic acid in human glycerides is higher than in the mouse. The phospholipid fractions of both the mouse and human show a general similarity in fatty acid composition. Content of linoleic acid is greater in the mouse than in human phospholipids. In general this comparison shows a significantly higher content of polyunsaturated fatty acids in mouse lipids than in the human.

Since all mouse plasma lipids are transported bound either to lipoproteins or to albumin the fatty acid composition of such macromolecular complexes was determined. In Table III are presented the data on fatty acids in 3 mouse plasma macromolecular fractions. Comparison of these data indicates the following points: (1) the low density lipoprotein fatty acid composition is very similar to the albumin-bound NEFA composition, (2) the percentage of arachidonic and stearic acid is highest in the HDL fraction, and (3) the percentage of oleate is highest in the low density lipoprotein fraction. From data obtained in this laboratory, it is known that approximately two-thirds of the lipid present in mouse plasma is generally carried in the HDL lipoproteins. Thus, the fatty acid composition of mouse plasma is in great part determined by the fatty acid distribution in HDL lipoproteins.

TABLE III. Fatty Acid Composition of Lipoprotein Fractions of Mouse Plasma. Values expressed as percentage of total fatty acid methyl esters.

Fatty acid	Lipoproteins		
	Low density	High density	Albumin-bound NEFA
Pre 16:0	.8	.4	1.2
Palmitic 16:0	25.0	18.1	28.0
Palmitoleic 16:1	2.3	1.0	2.9
16:1 - 18:0	.9	.9	1.3
Stearic 18:0	5.0	10.7	5.9
Oleic 18:1	24.1	8.9	19.8
Linoleic 18:2	33.8	39.3	32.5
18:2 - 20:4	3.9	3.4	4.0
Arachidonic 20:4	3.6	16.4	3.4
Post 20:4	.8	1.0	1.2

During these analyses it was found that mice normally transport a significantly higher amount of albumin-bound NEFA than humans. Values ranging from approximately 1330 to 2000 μEq/L are obtained in the mouse while in humans the values usually encountered are in the range of approximately 400 to 600 μEq/L. Since salt addition in the ultracentrifugal procedures used is known to displace a small amount of fatty acids from the albumin the above values are probably even somewhat less than actually present on mouse albumin.

Summary. 1. Mouse plasma lipid and lipoprotein fatty acids were evaluated by gas chromatography. 2. The plasma cholesteryl ester fraction contained a high percentage of polyunsaturated acids, linoleic (50.1%) and arachidonic (29.9%). 3. Mouse fatty acid data were compared with human values. 4. Concentration of albumin-bound NEFA in mouse plasma was found to be significantly higher than normally present in humans.

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Effect of pH on Muscle Calcium and Magnesium.* (26399)

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It has been pointed out that in maintenance of plasma pH, the role of the ion movement across cell membranes is not to be ignored(1). The relationship of permeating ion concentration to the membrane potential can be expressed by the following equation (2): $\frac{C_1}{C_2} = r^z q$. In this equation, subscripts 1 and 2 refer respectively, to intracellular and extracellular compartments; C is concentration of the ion; z = valence of the ion; $r = \text{antiln} \frac{F}{RT} E_m$ (F is the Faraday constant, R is the gas constant, and E_m is the membrane potential or difference in electrical potential of the extracellular compartment minus the intracellular compartment); and $q = \text{antiln} \frac{W_q}{R_T}$ (W_q is the active ion transport potential difference, *i.e.*, extracellular minus intracellular). Addition of acid causes the net valence of the intracellular non-permeating ions to increase by accepting the hydrogen ions, and this in turn causes the value of r to decrease(3). If extracellular ion concentration, C_2, and the ion work factor, q, are both constant, then intracellullar concentration, C_1, should be proportional to r^z. Thus, it is to be expected that acid addition to muscle cells should cause a decrease in intracellular concentration of the ion if the ion is a cation (*i.e.*, z is positive) and similarly alkali addition should cause an increase in intracellular concentration of the cation. If water shifts are ignored, then an increase in intracellular concentration indicates a cation shift into the intracellular water phase, and likewise a decrease in intracellular concentration indicates a cation shift out of the intracellular water phase. Thus, the cation would buffer the extracellular phase by exchanging with the hydrogen ion across the cell membrane. The purpose of this study was to determine if changes in extracellular pH would cause the expected shifts of muscle calcium and magnesium.

Methods. Frog muscles were dissected out and were maintained overnight in the control solution at 5°C. Generally, a muscle set consisted of the tibialis anticus longus, ileofibularis, semitendinosus, and the sartorius (occasionally the peroneus). Then one muscle set and its contralateral set were transferred respectively to the experimental and the control flasks, each of which contained 50 ml of solution. Tris(hydroxymethyl)-aminomethane was used as a buffer(4). All solutions contained 2 mM of $CaCl_2$, 2 mM of $MgCl_2$, 65 mM of NaCl, 2.7 mM of KCl, and 50 mM of tris(hydroxymethyl) aminomethane (Sigma 7-9 buffer, obtained from Sigma Chemical Co., St. Louis, Mo.). The pH was regulated by additions of various amounts of HCl. Five solutions were prepared which contained 60, 50, 40, 30, or 0 millimoles HCl per liter. The resulting pH of these solutions was 2.2, 6.8, 7.4 (control solution), 8.0, and 10.0, respectively. After 5 hours with gentle agitation at a temperature of about 23-26°C, the individual muscle sets were blotted on filter paper moistened with the control solution and weighed on a torsion balance. Each muscle set was placed in a platinum crucible, dried at about 100°C for approximately 1 hour, ashed overnight at 500°C, and dissolved in HCl. In one set of experiments, after precipitating with ammonium oxalate, calcium content was determined by an ethylenediamine tetraacetic acid (EDTA) titration using a calcium indicator (5). In another set of experiments, after precipitating with oxine, magnesium content and an approximate calcium (referred to as metal) content were determined by an EDTA titration using both a calcium and

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TABLE I. Effect of pH on Calcium and Magnesium in Muscle.

Muscle analysis	Exp. pH*	Millimoles/kg wt — Control	Experimental	Exp./Control, %	"P" value, %
Calcium	2.2	2.42 ± .04	1.55 ± .02	63.9 ± 1.46	.0
	6.8	2.30 ± .03	2.18 ± .02	94.9 ± 1.54	.7
	8.0	2.28 ± .05	2.35 ± .05	103.1 ± .95	.8
	10.0	2.38 ± .05	5.87 ± .08	247.5 ± 2.96	.0
Magnesium	2.2	6.52 ± .05	1.67 ± .04	25.7 ± .67	.0
	6.8	6.08 ± .04	5.10 ± .04	83.9 ± .72	.0
	8.0	6.15 ± .06	6.64 ± .06	108.4 ± .93	.0
	10.0	6.34 ± .04	7.76 ± .07	123.3 ± .60	.0
Metal	2.2	2.25 ± .07	1.42 ± .06	63.7 ± 3.24	.0
	6.8	2.28 ± .09	2.35 ± .11	103.3 ± 3.77	39.8
	8.0	2.35 ± .09	2.29 ± .11	98.1 ± 4.53	68.3
	10.0	2.17 ± .08	5.96 ± .35	280.6 ± 1.98	.0

* No. of experiments for each pH was equal to 12.

magnesium indicator(5,6). "P" values were obtained by comparing the per cent experimental/control values with 100%, and using a previously described "t" table(7).

Results. Results are given in Table I. The low pH of 2.2 caused a decrease of 4.85 millimoles magnesium/kg muscle and a decrease of only 0.87 millimoles calcium/kg muscle. On the other hand, the high pH of 10.0 caused an increase of 3.49 millimoles calcium/kg muscle and an increase of only 1.42 millimoles magnesium/kg muscle. Although the differences were not as pronounced for the 6.8 and 8.0 pH experiments, the results show that in experiments with a pH below 7.4 there was a decreased calcium and magnesium in the muscle, and in experiments with a pH above 7.4, there was an increased calcium and magnesium in the muscle. The metal determinations are used as confirmatory evidence for the calcium values.

Discussion. These experiments point out that both calcium and magnesium ions in muscle can contribute to the pH buffering of the extracellular phase by moving out of the muscle when the extracellular phase is acid and by moving into the muscle when the extracellular phase is alkaline. In solutions of moderate pH changes, calcium and magnesium shifts are not too pronounced, and probably other ions are quantitatively more important in tissue buffering. There appears to be more than one state of calcium(2,8) and magnesium(6) in muscle tissue and the main ion exchanges may not involve the intracellular water phase. In the case of calcium, Swan recently observed that acid solutions caused an efflux of Ca^{45} from muscle, which he interpreted to be due to an exchange taking place on the surface of the muscle(9). In reference to magnesium, previous studies have shown that only about one-fourth of the intracellular magnesium is exchangeable with Mg^{28}, a fraction which includes the free magnesium ion in the intracellular water(6). Since a lowering of pH to 2.2 has been found to release some 75% of the intracellular magnesium, we have clear support for the inference that extracellular acidity is capable of freeing magnesium from its non-exchangeable phase. On the other hand, it appears that the high pH of 10 was acting significantly to increase the binding of calcium. The failure of these 2 divalent ions to exhibit quantitatively equivalent shifts with changes in pH possibly relates in part to the different binding properties of the *in vivo* binding sites for these ions. Microincineration studies have been interpreted to indicate that calcium and magnesium are intimately associated with the contractile machinery of the myofibril(10). Perhaps this location represents a part of the non-exchangeable phase of these ions.

It should also be recognized that there appear to be active transport mechanisms for both calcium(2,11) and magnesium(6) and it is possible that changes in pH might influence this transport mechanism. However, measurements of membrane potential, ion flux, and ionic concentration of the various phases would be required to dissociate quantitatively this possibility from the conse-

quences of ionic binding in the various muscle phases.

Summary. These *in vitro* experiments show that both magnesium and calcium in muscle can contribute to the buffering of pH. In low pH solutions there is a decrease in both calcium and magnesium, and in high pH solutions there is an increase in both calcium and magnesium in muscle.

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Measurement of the Vascular Factor Attending Drug-Altered Sensitivity to Peptic Digestion.* (26400)

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The susceptibility of esophageal, gastric, and duodenal mucosa to acid peptic digestion depends upon pH and pepsin concentration of the gastric juice, the mucous barrier, tissue vascularity, and inherent cellular resistance. Clinical and experimental evidence has shown abetment of the acid peptic ulcer diathesis by various drugs including reserpine(1), serotonin, cortisone(2), pitressin (3) and nitroglycerin(4). It has been shown recently that reserpine increases susceptibility of the esophageal mucosa to acid peptic digestion(5).

The first segment of the experiment concerned assessment of the role of serotonin, ACTH(6) and hydrocortisone(7,8) which are released by reserpine, in the augmented sensitivity of the esophagus to injury by acid peptic juice. Pitressin, nitroglycerin, and chlorpromazine also were evaluated in this respect.

In the second segment of the experiment, the effect of reserpine, chlorpromazine, pitressin, and nitroglycerin on local esophageal blood flow was measured in an attempt to evaluate the importance of the vascular factor in potentiation of acid peptic digestion. A modification of Saperstein's technic was utilized(9).

Method. Adult cats under pentobarbital anesthesia (30 mg/kg) were used. Respiration was maintained by means of an automatic respirator through a cervical tracheostomy. In the first portion of the study, the animals were divided into 7 groups. The technic of perfusion, with different segments of the same cat's esophagus as the control and test preparation was used(5). In Group 5A, however, separate cats were used for control and test preparation. In the latter group, the perfusions using the same patient's gastric juice were started immediately after injecting pitressin intravenously into the test animal. At the end of the procedure, the mucosal surface of the esophagus was inspected. On removal of the esophagus, degree of injury to both segments was graded

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TABLE I. Drug Dosage Schedule and Comparison of Digestion of Control and Test Segments of Esophagus.

Group	No. of cats	Drugs	Dosage	Grade of digestion: Control 0	1	2	3	4	5	Test 0	1	2	3	4	5
I	6	ACTH	25 mg	2	2	2				1	2	3			
II	6	Hydrocortisone	10 "	3	1	2				1	3	2			
III	9	"	50 "	2	3	4				2			2	5	
IV	6	Serotonin	10 "	2	3	1				2	2	2			
V	9	Pitressin	20 units	5	1	2						3	3	3	
V a	12 pr	"	20 " *	2	6	3	1			1	2	1		5	3
VI	8	Nitroglycerin†		6	2					1	1	3		2	1
VII	3	Thorazine	10 mg	1		1	1				1	1		1	
	3	"	25 "	1	1	1				1	1	1			
	3	"	50 "	1	1	1					1	2			

* Perfusions started immediately after cats given pitressin.
† Because of short action, drug given: .65 mg I.M. & I.V. at start of test perfusion and at 60 min. with a .65 mg I.M. at 30 and 90 min.

0 to 5+ (0 : no digestion; 5+ : perforation). All perfusions were done at a pressure of 20 cm and a temperature of 37°C.

In the second segment of the experiment, all cats were prepared with cervical tracheostomy for maintenance of positive pressure respiration, cannulation of the right carotid artery and femoral vein, and exposure of the upper esophagus through a left thoracotomy. The animals were divided into 5 groups according to the intravenous medication: 1) 1 cc sterile H_2O; 2) 0.5 mg reserpine; 3) 25 mg chlorpromazine; 4) 20 u pitressin; 5) 0.65 mg nitroglycerin (intramuscularly and intravenously as in part I).

After a 2 hour period for drug action, arterial blood was taken for hemoglobin determination. Then 60 microcuries of K_{42} was injected rapidly into the femoral vein and simultaneously a continuous flow of blood from the arterial catheter was absorbed on a strip of No. 1 chromatography paper moving at 10 mm/sec. At least 30 seconds after K_{42} injection, a full thickness segment of esophagus was excised and placed in a weighed cuvette. Separate segments of the paper strip representing 2 seconds and the esophageal specimen were counted for gamma activity in a well-counter. The paper segments were analyzed for hemoglobin concentration, and each related to the original hemoglobin value. Number of counts per cc of blood and number of counts per gram esophageal tissue were then calculated. The area beneath the arterial isotope flow curve was determined. Flow in the esophagus was calculated by dividing number of counts per gram by this area, using the principle of the conservation of material (the present method assumes no K_{42} in venous return).

Results. ACTH, serotonin, 10 mg of hydrocortisone and chlorpromazine in doses of 10, 25, and 50 mg produced essentially no change in esophageal sensitivity to peptic perfusion. However, with nitroglycerin and pitressin, and the larger dose of hydrocortisone, marked increased susceptibility to peptic digestion was observed, nitroglycerin producing one perforation and pitressin, 3 perforations. In fact, 75%, 77%, and 66% of the nitroglycerin, single pitressin and paired pitressin-treated esophagi respectively showed greater than 2+ digestion of the drug-treated over the control side (Table I).

Administration of reserpine, pitressin, and nitroglycerin were followed by observed decreases in esophageal blood flow of 63, 35 and 70% respectively. Esophageal blood flow was not changed by chlorpromazine. Cardiac output in reserpine and chlorpromazine groups was similar to the control, whereas pitressin and nitroglycerin produced a 10 and 20% fall in mean cardiac output, respectively.

Discussion. The mechanism of increased susceptibility of esophageal mucosa to acid peptic perfusion induced by reserpine is obscure. A possible explanation is that the endogenous substances released upon reserpine administration are the toxic agents respon-

TABLE II. Esophageal Blood Flow (cc/g/min.).

Group	Control (I)	Reserpine (II)	Chlorpromazine (III)	Pitressin (IV)	Nitroglycerin (V)
No. cats	15	15	11	11	16
Spec. No. 1	1.10	.56	.56	.83	.40
2	.91	.43	5.28	1.45	.25
3	2.93	.28	1.75	1.29	.68
4	2.14	.90	.22	2.02	.69
5	2.19	.72	1.18	.72	.48
6	2.62	.51	1.87	1.38	.43
7	1.00	.48	1.10	1.26	.22
8	1.62	.28	1.65	.77	.55
9	.90	.28	.99	.81	.33
10	1.07	.29	.69	.62	.52
11	1.51	.85	1.25	1.13	.37
12	1.12	1.26			.96
13	1.07	.70			1.31
14	1.76	.80			1.46
15	2.10	.71			.46
16					.24
Mean $\bar{x}$	1.60	.60	1.50	1.12	.58
S.E. $\bar{x}$	.17	.07	.41	.13	.09
P value compared to control		$<<.001$	$>.40$	$>.02$	$<<.001$

sible for lowered tissue resistance. This possibility seems to have been excluded by the present study. A more likely mechanism could be a change of vascular flow resulting in tissue hypoxia, which makes the mucosa vulnerable to digestion. Several previous observations support this contention. Decreased catechol amines in the peripheral vessels, with subsequent increased sensitivity to sympathetic discharge, is known to occur as a reaction to reserpine(10,11). A peripheral vascular alteration has previously been proposed showing potentiation of peptic ulcer formation in cats and dogs given pitressin and nitroglycerin in addition to histamine in beeswax(3,4). Lowered tissue blood flow seems to be the common pathway of enhanced mucosal susceptibility to acid peptic digestion following administration of reserpine, pitressin and nitroglycerin. The possibility of a direct effect of these drugs on cellular metabolism is not excluded; however, the large flow changes observed suggest this as the more likely explanation. Chlorpromazine does not alter local esophageal blood flow nor enhance acid peptic digestion of the esophagus.

Conclusions. Tissue resistance to peptic digestion is decreased significantly by pitressin and nitroglycerin and by large doses of hydrocortisone. Reserpine, pitressin and nitroglycerin produced decreased esophageal blood flow. These experiments suggest definitely that decrease in blood flow abets the ulcer diathesis.

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Fiber and Pectin in the Diet and Serum Cholesterol Concentration in Man.* (26401)

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Some populations characterized by low serum cholesterol values subsist on diets high in fiber or cellulose and low in saturated fats, and it has been suggested that fiber may play a role(1,2). Our work in Italy(3,4) yielded somewhat lower serum cholesterol values than seemed to correspond with our findings in controlled experiments in man when comparisons were based solely on dietary fats (5). This discrepancy was confirmed in detailed analyses of data from Italy by means of the prediction formula, based on dietary fatty acids, developed from many controlled diet experiments on man(6,7).

Accordingly, direct controlled experiments were made in which groups of men subsisted alternately on "American" and "Italian" types of diet, comparable in calories, proteins and in kind and amounts of fats but differing in the sources of carbohydrates, an abundance of fruits and vegetables in the Italian types replacing equivalent calories in simpler carbohydrates in the American types. Lower serum cholesterol levels were consistently found with the Italian diets and the differences, though not large, were statistically significant(8).

We noted that the Italian type diets "tend to be high in complex carbohydrates such as pectin, hemicelluloses and fiber"(8), so new experiments were devised to examine the possible effects of these variables. This paper presents the results. The general nature of the findings was reported in abstract form to the Council on Arteriosclerosis of American Heart Assn.(9).

Subjects, methods and procedure. Methods and procedures were the same as in experiments at the Hastings State Hospital previously reported(5-9), and the subjects were similar, *i.e.*, physically healthy, middle-aged, male mental patients maintained under completely controlled conditions in a metabolic unit. In each experiment men were maintained on a standard diet for a stabilization period of 3 weeks, then were assigned to dietary sub-groups matched as to age, relative body weight (obesity), activity habits and general level of serum cholesterol. Thereafter, for successive 3-week periods, the sub-groups subsisted on the experimental diets in a switch-back or reversal pattern so devised as to compensate for possible time trends. The design of the present experiments is summarized in Table I.

One series of experiments tested the effect of fiber or cellulose with each of 2 different diets and another tested the effect of pectin with each of 2 somewhat different diets. Blood samples were drawn from an arm vein from each man on 2 occasions at the end of each dietary period and the serum was analyzed for total cholesterol, in duplicate, by our modification of the method of Abell *et al.*(10).

Diets. Each diet was composed of natural foods in a rotating series of 7 daily menus of 3 meals each corresponding with ordinary American customs. All of the diets were closely similar in calories, fats and cholesterol but they formed 2 pairs. The diets in the S pair of experiments differed in the source of part of the carbohydrate, bread and potatoes in Diet SB being replaced in part in Diet SS by equal calories in sucrose. In the U pair of experiments, Diet UB provided a considerable amount of carbohydrate in legumes which was isocalorically matched in sugar in Diet US. The small differences in proteins resulting from these food source dif-

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TABLE I. Design of Experiments S and U. Diets fed during days indicated. In each case the same standard stabilization diet was fed during days 1-21. "C" = Alphacel, "P" = Pectin, N.F.

Group	No. men	Days 22-42	Days 43-63	Days 64-84	Days 85-105
Q	6	SB	SB + C	—	—
R	6	SB + C	SB	—	—
S	7	SS	SS + C	—	—
T	6	SS + C	SS	—	—
W	6	UB	UB + P	US + P	US
X	6	UB + P	UB	US	US + P
Y	6	US	US + P	UB + P	UB
Z	6	US + P	US	UB	UB + P

ferences were equalized by the use of wheat gluten and soy bean protein concentrate. The food items selected assured an abundance and relative constancy of vitamins and minerals in all diets. Variability in the foods themselves was avoided by laying in sufficient stocks of staples before the start of each experiment.

The nutrient contents of these diets were estimated from standard food tables. Individual differences in calorie requirements were observed during the pre-experiment stabilization periods and individual allowances were made on this basis, adjustments being made in amounts of simple carbohydrate foods provided (bread, sweets). All servings were individually measured and rejections and plate waste were recorded. Individual allowances were further adjusted when indicated from the body weights (nude) measured at frequent intervals.

A constant, closely supervised regimen of rest, exercise and recreation helped to maintain a high degree of calorie constancy. This is indicated by the actual calorie intakes and body weight data in Table II, which also shows the close correspondence between intakes of all nutrients in the basal diets and the cellulose or pectin supplemented periods with those diets.

The effects of cellulose (fiber) and of pectin were judged by comparing the same men on the same diet with and without additions of these substances at the level of 15 g daily. Cellulose ("Alphacel") was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. Pectin N. F. (U. S. National Formulary) was donated by Sunkist Growers Co., Ontario, Calif. (Pure Citrus Pectin, lot 444-H). The "Alphacel" and pectin were incorporated in special biscuits. During periods when these supplements were not given the men ate biscuits similar in appearance and equivalent in calories.

Results. Each sub-group consisted initially of 7 men but during the experiments a few men were dropped for medical reasons, failure in cooperation or excessive variability in

TABLE II. Mean Nutrients Actually Consumed Daily during Dietary Periods Indicated in Table I. Sat., mono., and poly. refer to glycerides of saturated, monoene and polyene fatty acids. "CHO" = carbohydrate. Mean body wt pertain to the end of the periods on diets indicated by column headings. "Veg." = leafy vegetables only.

Item	SB	SB + C	SS	SS + C	UB	UB + P	US	US + P
Mean body wt, kg	69.4	69.5	67.5	67.6	70.4	70.4	70.2	70.2
Calories	3060	3025	3043	3104	3025	3038	3055	3038
Proteins, g	97	97	96	97	124	123	125	124
Total fat, g	138	137	138	139	140	140	137	137
CHO in sucrose, g	28	28	155	164	19	19	142	142
" " legumes, g	4	4	4	4	118	118	3	3
" " veg., g	2	2	2	2	5	5	5	5
" " fruits, g	35	35	35	35	21	21	21	21
Fiber, g	5	20	5	19	10	10	10	10
Cholesterol, mg	569	564	570	571	577	576	577	575
% cal. from sat.	19.7	19.8	19.9	19.7	19.9	19.8	19.7	19.8
" " " mono.	17.6	17.5	17.6	17.5	17.8	17.8	17.7	17.7
" " " poly.	1.9	1.9	1.9	1.9	2.0	2.0	2.0	2.0

TABLE III. Dietary Fiber (Cellulose). Mean serum cholesterol, mg/100 ml, at the ends of dietary periods. △ and stand. error (S.E.) values calculated from the individual differences on the diets compared. "C" = added cellulose.

Groups	No. men	Diet type	Serum cholesterol, mg % No C	+ C	△	S.E.
Q, R	12	SB	204.8	212.9	8.1	± 5.0
S, T	13	SS	234.4	236.7	2.3	± 4.1

Exp. SB, t = 1.62, p = >.1
" SS, t = .56, p = >.5

eating, etc. The others remained well and showed no variations in behavior. Serum cholesterol findings are summarized in Tables III and IV.

Table III shows no effect of the cellulose supplement in either of the diets in which it was tested. Contrary to some speculations (1,2) there is no tendency for serum cholesterol concentration to fall when the diet is high in fiber or cellulose. Actually, in both experiments mean cholesterol value rose slightly but the change is not statistically significant.

Table IV shows a small but statistically significant decline in serum cholesterol concentration in both experiments when pectin was added to the diet. The average effect, a fall of about 5% from the level without the pectin supplement, is less than previously observed in comparisons of Italian and American type diets. From this it might be inferred that only a part of the differences observed on these diets could be ascribed to differences in their pectin content.

Discussion. A recent study, reported in abstract, indicates that pectin amounting to 5% of the diet fed to very young rats had no effect on cholesterol level in either plasma or liver unless the diet also included one per cent cholesterol, in which case the pectin seemed to counteract some of the cholesterol rise which occurred otherwise(11). Five per cent of the (dry) diet as pectin is of the order of twice the dosage used in the present experiments. Because of major differences among species in cholesterol metabolism, no extrapolation of these findings to man is justifiable.

TABLE IV. Dietary Pectin. Mean serum cholesterol, mg/100 ml, at the ends of dietary periods. △ and stand. error (S.E.) values calculated from individual differences on the diets compared. "P" = added pectin.

Groups	No. men	Diet type	Serum cholesterol, mg % No P	+ P	△	S.E.
W,X,Y,Z	24	UB	202.4	192.7	− 9.7	± 2.6
W,X,Y,Z	24	US	221.5	211.3	−10.2	± 4.3

Exp. UB, t = 3.72, p = .001
" US, t = 2.40, p = .02

The paucity of data on fiber and particularly on pectin contents of human diets makes it difficult to relate the present experiments to diets as eaten naturally. The amount of cellulose used here is certainly higher than commonly ingested in so-called civilized diets. In the men at Hastings the cellulose supplement was well tolerated and no serious diarrhea resulted but we concluded that larger dosage would not be desirable. Alterations in gastro-intestinal function were clearly shown by changes in motility revealed by serial roentgenograms.

Calculations from such rough data as are available(12,13) suggest that 20 g of cellulose or fiber daily would seldom be attained in diets in Italy or even in the Bantu diets we studied in South Africa(14). It seems, therefore, unlikely that dietary fiber or cellulose plays any significant role in producing low serum cholesterol levels observed in Italy (13) or among the Bantu(14).

There are no reliable estimates of the amount of pectin in human diets. Pectin is present in most fruits and berries, particularly in apples, citrus fruits, and all fruits and berries notable for their value in making jellies. In citrus fruits most of the pectin is in the white inner rind and the connective tissue so it is largely lost as these fruits are ordinarily eaten, particularly in the form of juice. The largest usual source of pectin in human diets is probably in apples but 15 g daily, as used here, would be obtained from this source only by a most enthusiastic apple eater.

It is possible, of course, that more prolonged subsistence on diets containing large amounts of fiber or of pectin would have results different from those reported here.

However, the effects of dietary fats are largely or wholly achieved in a few weeks, with little or no subsequent change.

Summary. Rigidly controlled experiments on middle-aged men subsisting on diets of natural foods with and without supplements of 15 g daily of either cellulose (fiber) or pectin failed to show any significant effect on serum cholesterol concentration from the cellulose but they did consistently show an effect from the pectin. The pectin effect was apparent in 3 weeks and amounted to an average fall of about 5% below the level on the same diet without pectin supplement. It is suggested that the amounts of cellulose and pectin used correspond to the upper levels of these substances provided in natural human diets.

We are grateful to Mrs. Helen Williams for supervising the dietary work and to Mrs. Nedra Foster for aid in devising diets as well as supervision of the technicians responsible for cholesterol and food analyses. This work would have been impossible without the cooperation of Dr. William F. Sheeley, Supt. of Hastings State Hospital, and the devoted assistance of volunteer aides from the Church of the Brethren: Gary McCann, Richard W. Roller, John Hall and John Buehrer.

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Gonadotropin Secretion in Lactating Mice.* (26402)

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Ovariectomized mice, bearing intraocular ovarian transplants, show normal vaginal cycles and repeated development of vesicular follicles and white corpora lutea(1). Similar hosts, with intraocular pituitary transplants in addition, exhibit pseudopregnancy cycles (2), as observed in intact mice with pituitary isotransplants(3). These cycles are correlated with the development of red corpora lutea in the ovarian transplants. The corpora, unlike those in hosts without pituitary transplants, are functional.

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In the present study, ovarian transplants were placed intraocularly in lactating mice which were simultaneously ovariectomized. Transplants were observed throughout the lactation period for evidence of the action of follicle stimulating (FSH), luteinizing (LH) and luteotrophic (LTH) hormone; an attempt was made to prolong the period by substituting suckling young with newborn mice when the former were 2 weeks of age.

Material and methods. Twenty BALB/c mice, each of which had delivered a litter within the same 48 hour period, were matched with 20 virgin females of the same

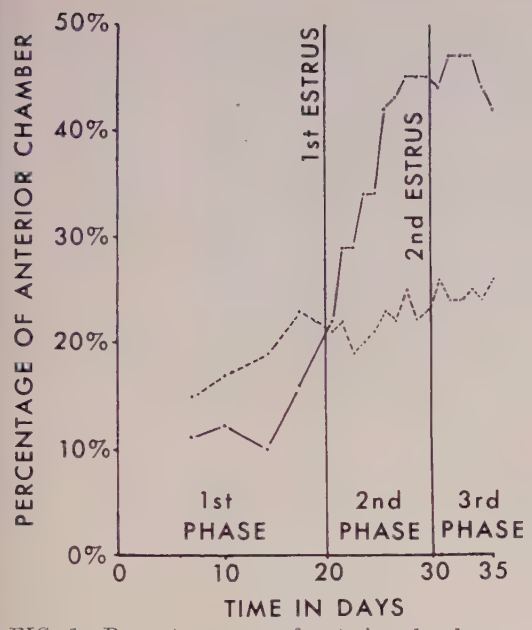


FIG. 1. Percentage area of anterior chamber occupied by ovarian transplants in lactating (———) and non-lactating (– – – –) ovariectomized mice (18 transplants/group) with mean time of appearance of the first and second estrus in lactating mice.

age and strain. The lactating females were castrated within 24 hours of parturition, and each immediately received an isotransplant of ⅛ of an adult ovary, from a donor of the same age, in the anterior chamber of each eye. The virgin females were treated similarly, each within 10 minutes of the corresponding lactating female.

Transplants were observed from the end of the 1st week, and vaginal smears were taken daily (by lavage) from time of transplantation, until the 35th day when experiment was terminated. Lactating mice with litters of less than four were rejected. Litters of the remaining mice were replaced at the end of the 2nd and 4th weeks with the original number of newborn mice (4 to 8). Representative females were sacrificed at intervals and their intraocular grafts removed for histological purposes. Nine pairs of animals completed the experimental period.

Results. In virgin females, intraocular grafts had become vascularized and were growing by the end of the 1st week. The average amount of anterior chamber occupied, in surface area, was 15% on the 7th day, 23% by the 17th day, and only 26% at termination of experiment (Fig. 1).

By the 10th day, all transplants showed vesicular follicles and, by the 14th to 17th days, corpora lutea; thereafter cyclic development of the two occurred. Vaginal smears sometimes showed uninterrupted estrous cycles after ovariectomy and transplantation but often exhibited a prolonged diestrus and erratic cycles, especially in the first 20 days. The corpora lutea, newly formed after estrus, occasionally had a pink color. Such corpora often occurred in only one anterior chamber, those in the opposite eye having the usual opaque white appearance. The pink corpora lasted usually only one day, with immediate reversion to the white type (Fig. 2). Mean length of 10 estrous cycles occurring after the 20th day was 7.7 days, with an associated diestrous interval of 3 days.

In lactating females, growth and development exhibited 3 phases. The *first* occupied the initial 17 to 21 days after ovariectomy and transplantation. During this period, transplants resembled those in virgin females in vascularity and development of vesicular follicles, but the latter did not luteinize. Transplants occupied 11% of the anterior chambers at end of the 1st week and showed no growth by the end of the 2nd week; however, by the 17th day, they occupied 16% and, by the 20th day, 22%, having reached the size of those in virgin females.

Between the 17th and 21st days, the first estrus occurred, marking the beginning of the *second phase.* At estrus (or, occasionally, at proestrus or the day after estrus), the vesicular follicles of all transplants abruptly transformed into corpora lutea with a distinct pink or an intense red color (Fig. 2). Anastomosing vessels on the graft surfaces became engorged. All pink corpora became red after 1 to 3 days; red corpora persisted for 2 to 6 days. They then either reverted to the pink type for 1 to 3 days or, more usually, lost all coloration as abruptly as they had gained it, and resembled white corpora lutea in virgin females. The total

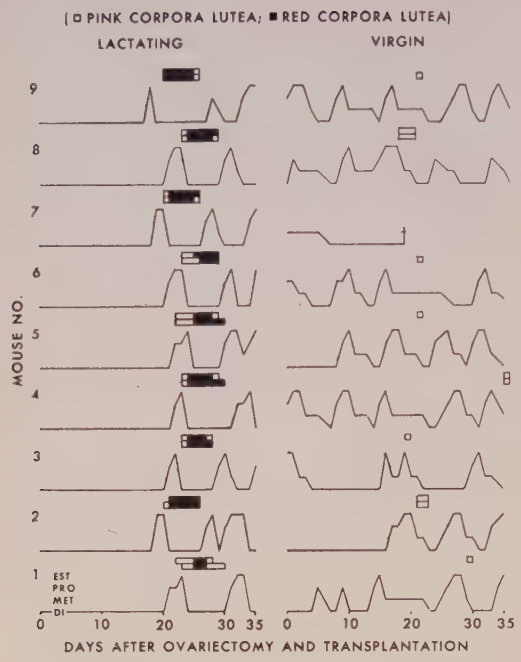


FIG. 2. Vaginal estrous cycles and time of appearance of pink or red corpora lutea in intraocular ovarian transplants in ovariectomized lactating or virgin mice.

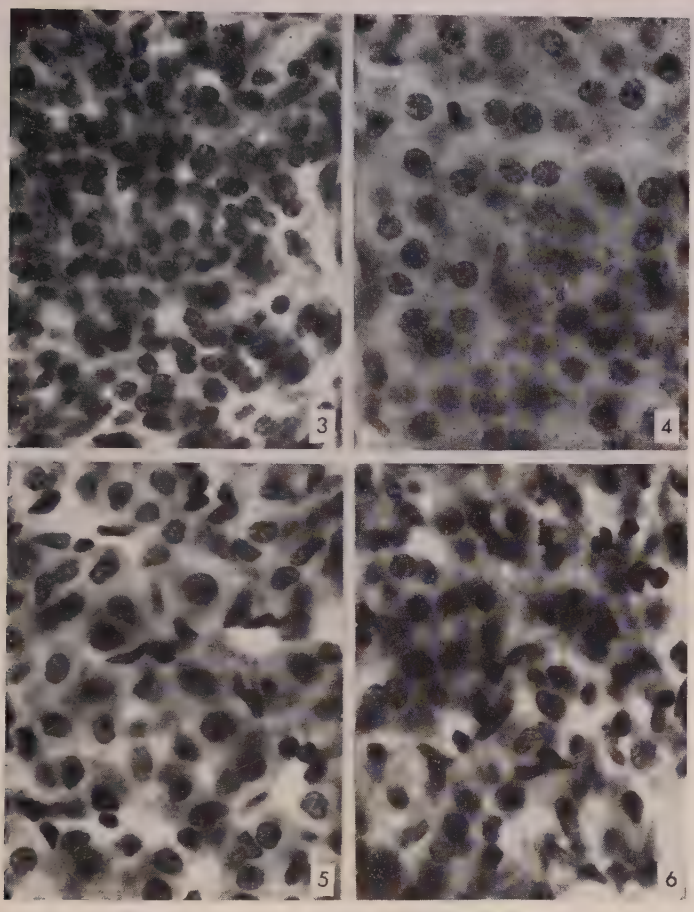
period during which corpora were pink or red was remarkably constant, being 6 days for 12 of the 18 transplants, 5 or 7 days for 5, and 8 days for the remaining one. During this second phase, vaginal smears were of the diestrous type, with a mean duration of 6.5 days and a range of 5 to 9 days. Concurrently with this phase, the surface area occupied by the intraocular grafts increased from 22% to 45% (Fig. 1).

On the 1st or 2nd day after reversion of the corpora to the white type (Fig. 2), a new estrous cycle commenced (except in one animal where it began on the last day of red corpora), marking the beginning of the *third phase*. The mean interval between vaginal estrus at the beginning of the second and third phases was 8.9 days. The corpora lutea regressed, slowly losing their distinct outlines, and were replaced by vesicular follicles and new corpora. The latter occasionally showed a pink color for one or 2 days. During this third phase, the females became unable to maintain their substituted litters. New estrous cycles began to follow without development of red corpora lutea or a prolonged diestrus, ending the third phase. Transplants showed no further growth in this period.

Histologically, pink corpora lutea were composed of closely-packed cells with large nuclei and scanty cytoplasm (Fig. 3). They often showed mitotic activity, accompanied by an increase in amount of cytoplasm. Red corpora lutea consisted of cells with comparatively large amounts of cytoplasm, very uniform nuclei, and islands of red blood cells (Fig 4). After reversion to white corpora in the early part of the third phase, the cells still retained relatively abundant cytoplasm, but cell boundaries were unusually distinct, nuclei less uniform, and islands of red blood cells rare or absent (Fig. 5). As these corpora lutea regressed in the third phase, the cytoplasm diminished in amount and nuclei became irregular. Corpora lutea of virgin females resembled the pink corpora of lactating females, except for greater variability in nuclear size and extensive vacuolization of the cytoplasm (Fig. 6).

Discussion. The development of vesicular follicles in intraocular ovarian isotransplants in ovariectomized mice, in contrast to the minute immature follicles seen in such transplants in intact mice, indicates the action of pituitary FSH; luteinization, which follows vaginal estrus in the ovariectomized animals, indicates pituitary release of LH(1,4). Uterine deciduomata cannot be induced in similar animals, bearing pituitary as well as ovarian transplants, unless *red* corpora lutea are present. The red color, presumably due to sinusoidal engorgement, indicates LTH release by the pituitary transplant, with consequent progesterone secretion by the red corpora (2).

Inferentially, in the present study, pituitary release of FSH continued in lactating mice, for vesicular follicles developed in their intraocular ovarian transplants. However, in comparison with virgin mice, this release may have been at a low level, for appreciable growth was absent until late in the lactation interval. On the other hand, LH release did not occur, for the follicles did not luteinize as



Intraocular ovarian isografts in lactating or virgin female mice. ×665.

FIG. 3. Pink corpus luteum showing closely packed nuclei, indistinct cell boundaries and scanty cytoplasm; early *second phase*, lactating female.

FIG. 4. Red corpus luteum showing uniform nuclei, abundant granular cytoplasm, and areas of red blood cells; *second phase*, lactating female.

FIG. 5. White corpus luteum, showing irregular nuclei, distinct cell boundaries and absence of red blood cells; early *third phase*, lactating female.

FIG. 6. White corpus luteum, showing very variable nuclear size and extensive cytoplasmic vacuolization; *cyclic* virgin female.

they did in virgins. Presumably, LTH was being secreted continuously in relation to lactation but, in the absence of luteinized follicles, its luteotropic effects could not be expressed. As the end of the lactation interval approached, LH release occurred, for corpora lutea developed and were immediately converted into the functional (red) type by the pre-existing LTH. At the same time, FSH release was also stepped up for transplants showed very active growth in this period (end of 1st phase).

Rothchild(5,6) considered that both FSH and LH were virtually absent during lactation, and pseudopregnancy, owing to suppression of their formation in the pituitary by the suckling stimulus in the former and to inhibition of their release from the pituitary by progesterone in the latter. The present findings are compatible with this hypothesis although LH rather than both FSH and LH secretion was suppressed during the lactation interval (1st phase). In the subsequent pseudopregnancy (2nd phase) functional corpora lutea were present and their disappearance was followed by a new estrus. However,

exogenous progesterone(4) gave no evidence of inhibition of FSH or LH release in castrated female mice bearing ovarian transplants (although it did produce complete inhibition of such release in intact males with similar transplants). In any case, as has been suggested(7), the lactation interval is not under luteal control for no corpora lutea were present during the 1st phase.

Despite maintenance of a strong suckling stimulus by litter substitution, lactation began to fail after the end of the 1st month (beginning of the third phase), although lactation has been prolonged to 2 months in intact mice by the same technic(8). While the amount of ovarian tissue in bilateral intraocular transplants is considerably less than in the ovaries *in situ,* it is sufficient to reestablish estrous cycles in virgin mice. Further, only LTH and corticosteriods are essential for lactation(9) over short intervals at least; possibly prolonged lactation requires a higher level, or a different type, of ovarian hormone than the small and ectopic intraocular tissue can supply.

The occurrence of estrus in the 3rd week after parturition, ending a more or less constant "lactation interval," but with continued lactation through one or more "pseudopregnancy cycles," has been observed before (5, 8). The lactation interval evidently corresponds to the *first phase* of the present study; the *second phase,* with red corpora lutea, is the equivalent of a pseudopregnancy cycle. Such an event, with functional corpora lutea like those formed after the immediate postparturitional estrus of the intact mouse, and accompanied by a far longer period of uterine decidual reactivity than in gestation(10), seems to be coincidental with, rather than essential for, lactation.

Summary. 1. Intraocular ovarian isotransplants in ovariectomized lactating BALB/c mice showed little growth and developed only vesicular follicles in the first part of the lactation interval. The first vaginal estrus occurred between 17 and 21 days after parturition, followed by abrupt appearance of pink or red corpora lutea. 2. These atypical corpora persisted from 5 to 8 days, concurrently with vaginal diestrus. They then became white and a second vaginal estrus occurred, unaccompanied by red corpora. Transplants showed considerable growth between the first and second estrus. 3. Similar transplants in ovariectomized virgin females developed vesicular follicles and corpora lutea from the 10th day onwards, with more or less normal estrus cycles. Corpora were typically white. Growth of transplants was moderate but continuous. 4. It is suggested that red corpora lutea indicate the action of LTH; and that pituitary release of LH, but not of FSH, is inhibited over most of the lactation interval.

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Hemagglutination and Hemadsorption of Measles Virus. (26403)

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Since the first evidence of successful isolation and propagation of measles virus in human renal cells by Enders and Peebles(1) many laboratories have reported studies on growth properties, adaptation to other host tissues, as well as serological responses in humans and laboratory animals. Recently, Peries and Chany(2) have reported their findings on yet another property of this virus, namely, the phenomenon of hemagglutination. They found that hemagglutination occurred only with monkey blood cells and that this hemagglutination could be inhibited by measles convalescent serum but not by the serum of subjects without previous measles experience. This is to report further studies on agglutination of monkey red blood cells by different measles virus preparations and inhibition of this hemagglutination by various measles antisera.

Methods. The Edmonston strain of measles virus, kindly supplied by Dr. J. Enders, was used in this study. In some experiments an additional measles isolate, kindly supplied by Dr. J. Frankel, was used for comparison. Complement fixation (CF) tests were performed according to the technique described by Jensen(3).

Hemagglutination (HA) tests were performed by 2-fold serial dilutions of virus in physiological saline in a volume of 0.5 ml. Five-tenths (0.5) ml of 0.5% monkey red blood cells in saline was then added to all tubes. One HA unit of virus was considered to be the highest dilution of virus showing complete hemagglutination.

Hemagglutination-inhibition (HA-I) tests were performed by mixing 4 HA units of virus in a volume of 0.25 ml and 2-fold serial dilutions of serum in 0.25 ml. The mixtures were shaken thoroughly and incubated at room temperature for one hour. Five-tenths ml monkey red blood cells (0.5%) was then added and incubation continued. Tests were read when red cell controls and serum controls were negative. Reshaking and reincubation of tubes at room temperature overnight occasionally clarified end point reactions. The HA-I titer of a serum was considered to be the highest dilution of that serum which completely inhibited hemagglutination of 4 HA units of virus.

Concentration of hemagglutinin by centrifugation was accomplished by centrifuging different measles virus harvests in a Spinco, Model L, ultracentrifuge, in a No. 30 rotor. Sedimented debris (pellets) were reconstituted in one-tenth of the original starting volumes in saline. Pellets were found to reconstitute easily.

Results. Hemagglutination of measles virus was demonstrated with 10× concentrated measles virus specimens only when red blood cells from monkeys were used. Cells from rhesus, cynomolgus, patas, African green or baboon all were equally sensitive. In all subsequent tests red cells from African green monkeys were used. Red blood cells from 11 other mammals: sheep, chick, guinea pig, rabbit, human types AB and O, horse, hamster, mouse, rat, cat and cow were negative.

The ability of measles virus to agglutinate monkey red blood cells varied according to the tissue in which the virus was grown. Table I lists some of the preparations used, their approximate passage history and their infectivity, CF and HA titers. We have found that measles virus propagated in one passage of primary baboon kidney cells yields higher titered infectious measles virus preparations than when grown in human amnion or monkey kidney (rhesus or cynomolgus) cells. These preparations also showed higher HA titers than other preparations. Measles virus propagated in chick fibroblasts showed virtually no hemagglutination even though infectivity titers were between $10^{4.0}$ to $10^{4.7}$. Hemagglutination was observed, although to a lower titer, with the Edmonston strain of measles virus propagated in primary amnion

TABLE I. Comparison of Serological Reactions of Measles Preparations.

No.	Sample	Strain—known passage history	Reciprocal of titer INF.*	CF	HA†
1	MeM446BK	Edmonston—human amnion (prim.) baboon	6.0	256	32
2	MeM387BK	*Idem*	6.0	256	32
3	MeM38	Edmonston—human amnion (prim.)	3.5	N.T.	4
4	MeLB4-5	Edmonston—human amnion (prim.) chick embryo chick fibroblast	4.3	<4	<4
5	Me33G-D	Edmonston—human amnion (prim.) monkey kidney	3.0	N.T.	2
6	MeG.H. (live)‡	Edmonston—human kidney monkey kidney Girardi heart	approx. 4.0	64	8
7	MeG.H. (inactivated)§	Edmonston—human kidney monkey kidney Girardi heart	—	256	32
8	Me #6‖	Isolate —human amnion (cont.)	4.5	32	8

* Negative $\log_{10}$ $TCID_{50}$/ml.
† Highest dilution showing complete hemagglutination (10× concentrates).
‡ Prepared by Microbiological Associates, Inc.
§ Commercially available from Microbiological Associates, Inc.
‖ Kindly supplied by Dr. J. Frankel, Norristown State Hosp., Norristown, Pa.
N.T. = Not tested.

TABLE II. Concentration of Hemagglutinin Activity.

Sample	Treatment	Hemagglutination titer
Prep. 1 MeM38-7BK	(Centrifugation)	
a) Original sample	None	1/8
b) Supernate	11,000 rpm/90 min.	1/8
c) Pellet (10×)	*Idem*	1/32
d) " —7BK control (10×)	"	0
Prep. 2 MeM38-7BK	(Centrifugation)	
a) Original sample	None	1/8
b) Supernate	30,000 rpm/180 min.	<1/4
c) Pellet (10×)	*Idem*	1/64
d) " —7BK control (10×)	"	0

or with another measles isolate propagated in a continuous line of human amnion cells. Hemagglutination also was observed when killed measles antigen* or preparations containing live measles virus prepared in another laboratory were used. Hemagglutination was not seen in the original or concentrated suspensions of uninfected tissue cultures.

Ratios of infectious virus particles to hemagglutination units from 4 different measles concentrates (Table I) were similar and varied between 1.3 x 10^4 to 4.0 x 10^5.

* Microbiological Associates, Inc.

Preliminary studies have demonstrated that concentration of virus is necessary to demonstrate adequate titers of the hemagglutinin even on those preparations grown in baboon kidney (Table II). Centrifugation in a Spinco, Model L, centrifuge at 30,000 rpm/180 minutes concentrated the hemagglutinin so that hemagglutination inhibition tests could be satisfactorily performed. Hemagglutinin activity was found in uncentrifuged samples or supernatant samples of preparations centrifuged at only 11,000 rpm/90 minutes.

Stability studies on limited amounts of

TABLE III. Comparison of HA-I and CF Titers of Naturally Occurring Measles Antibodies in Simian Sera.

Animal	Sera #	Species	Reciprocal of titer CF	HA-I
Baboon	3	Papio unibas	256, 512	128
	11	" "	64	8
	6	Papio dougeriae	128, 128	128
	7	" "	64	64
	8	" "	32	64
	25	" "	128, 256	1024
	27	" "	64	128
	28	" "	64	64
African green monkey	9	Cercopithecus aethiops	64	8
	10	" "	128	128
Rhesus	Standard hyp. serum	Macaca mulatta	256	1024
Baboon	Each of 24 sera	Papio unibas	<8	<8
African green monkey	Each of 19 sera	Cercopithecus aethiops	<8	<8

10× concentrated measles preparations have shown that all hemagglutinin activity is lost in 2 hours at 56°C. There is loss after 3 to 4 days at 37°C, but the hemagglutinin is stable for at least 7 days at –20°C and 5°C, and for at least 14 days at –70°C.

Incubation of hemagglutinated measles virus at 37°C for periods up to 36 hours have shown that elution of the virus as evidenced by sedimentation of red cells did not occur. The use of tanned red blood cells according to the technique described by Arbesman, *et al.* (4), did not significantly increase the sensitivity of the hemagglutination reaction.

Infected stationary tubes of various cell types infected with measles virus were also examined for direct tissue culture hemadsorption. Areas of positive hemadsorption patterns in measles inoculated kidney cultures were clearly evident, whereas control cultures were negative. When measles infected tubes were tested with red blood cells from animals other than monkeys, no positive patterns of hemadsorption could be seen. However, when these red cells were washed off and monkey cells added, positive hemadsorption again was noted.

A study was made of the HA-I titers in a limited number of sera from monkeys with naturally acquired measles, artificially induced hyperimmune measles sera, and human measles sera to determine whether correlation existed between measles antibodies from those sources.

Table III shows CF and HA-I titers in 10 simian sera against measles occurring naturally. These 10 sera contained CF as well as HA-I antibodies. A total of 43 negative simian sera by CF procedures were also negative by HA-I.

HA-I tests performed with hyperimmune measles antisera (Table IV) show that all sera obtained from animals immunized with measles antigens contained high levels of HA-I antibodies. Inhibition of hemagglutination by these antisera was observed when any antigen was used. Of particular interest is the fact that antisera prepared against measles virus grown in chick fibroblasts inhibited hemagglutination of measles virus even though little or no hemagglutinin activity could be demonstrated with chick grown virus. Specific human gamma globulin in high dilution also inhibited hemagglutination of measles virus prepared in our laboratory. Normal sera and sera from animals immunized with control tissue culture cells contained no antibodies.

Antisera against a number of viruses, mumps, respiratory syncytial, herpes simplex, parainfluenza, Newcastle disease, vaccinia, polio types 1, 2, and 3, influenza strains Lee and Jap 305, sendai, and canine distemper failed to inhibit measles hemagglutination.

Analyses of the HA-I and CF titers of 4 samples of paired human sera obtained in the Philadelphia area during an epidemic of measles in 1957, as well as 8 samples of convales-

TABLE IV. Hemagglutination-Inhibition Titers of Measles Hyperimmune Sera.

	Measles virus* grown in	Antisera prepared in	Reciprocal of highest dilution showing complete inhibition of hemagglutination†
1.	Girardi human heart‡	Monkey	2560
		Control monkey serum	8
2.	Chick embryo fibroblasts	Rabbit	256
		Control rabbit serum	<4
3.	Continuous human amnion	Rabbit	512
4.	*Idem*	Guinea pig	>4096
		Control guinea pig	8
5.	Baboon kidney	Guinea pig	4096
		Control guinea pig	16
6.	γ-globulin§	Human	6400

* Edmonston strain of measles.
† Against 4 HA units of concentrated measles virus.
‡ Killed CF antigen—Microbiological Associates, Inc.
§ American Cyanamid Co.

cent measles sera, are presented in Table V. Significant increases in HA-I antibodies were found in 3 out of 4 of the paired convalescent serum samples over that in the acute specimen. One matched pair showed only a 2-fold increase in titer in the convalescent serum over the acute samples. The 8 convalescent measles samples showed comparable CF and HA-I activity.

TABLE V. Comparison of Hemagglutination-Inhibition and CF Titers of Human Sera against Measles Virus.

Human sera No.	Titers† CF Acute	CF Conv.	HA-I Acute	HA-I Conv.
1	<8	128	8	256
2	8	256	N.A.‡	256
5	4	64	<8	64
7	<8	64	N.A.	512
11	<4	256	8	128
12	<8	128	64	128
60-2749* 1		256		32
2		256		32
60-1279* 1		256		256
2		128		256
60-1091*		64		512
60-1210* 1		128		256
2		128		512
60-1204*		64		128
Standard monkey hyp. sera	256		1024	

* Kindly supplied by Dr. K. Hummler, Children's Hospital, Philadelphia, Pa.
† Reciprocal of dilution.
‡ None available.

Discussion. An interesting observation was the apparent failure of chick TC grown measles to hemagglutinate. This was strikingly similar, at least in our hands, to the failure of these preparations to demonstrate complement-fixing antigen. CF measles antigen was demonstrated when primate tissue was used as the host tissue even when infectivity titrations showed that titers were below $10^{-4.0}$. Peries and Chany(2) have pointed out that in their system of KB infected measles cells, $10^{5.0}$ to $10^{6.0}$ infectious particles per ml must be attained in order to demonstrate hemagglutination. It might be that more potent chick grown measles virus preparations will demonstrate hemagglutination; however, it is conceivable that this virus present in supernatant fluids may be bound in such a manner that hemagglutination does not occur. These points are now being investigated.

As hemagglutination and hemadsorption are related manifestations in the case of certain other viruses, it was interesting to find that positive hemadsorption patterns could be observed with monkey red blood cells and tubes infected with measles virus. Hemadsorption technics, therefore, should offer a

more rapid means of attempting to isolate measles virus as well as studies on adaptation of the virus to other tissues.

The observations that CF and HA-I titers were comparable in measles sera from simian, rabbit, guinea pig, and human (the latter small in number), and that no CF or HA-I antibodies were found in a larger number of measles negative sera would indicate that hemagglutination-inhibition may be useful in evaluation of antigenic responses in man and animals, assay of virus pools, and standardization of hyperimmune sera.

Summary. Evidence has been presented to show that concentrated measles virus in presence of monkey red blood cells will demonstrate the phenomenon of hemagglutination. Baboon kidney propagated measles virus yielded higher levels of hemagglutinin than measles virus grown in other primate tissue. Chick tissue propagated measles virus did not demonstrate hemagglutination. Specific inhibition of hemagglutination was demonstrated by rabbit and guinea pig antisera prepared against measles virus in our laboratory and in another laboratory, by naturally occurring simian measles antisera, by human gamma globulin and by human convalescent measles antisera. Inhibition was not observed when measles CF negative simian sera, measles acute human sera and a number of viral antisera were tested.

ADDENDUM: After this paper was submitted for publication, an article entitled "Hemagglutination and Hemagglutination-Inhibition with Measles Virus" by Leon Rosen appeared in *Virology,* 1961, v13, 139. The work reported herein is in complete agreement with his observations.

Technical assistance of Robert Jakubowski is gratefully acknowledged.

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Experimental Growth of Mammary Gland in Male and Female Mice.* (26404)

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Estrogen (E.B.) and progesterone (P) (1,2) or relaxin(3) have been shown to synergize in stimulating lobule-alveolar growth in male and female mice in short-time experiments. Recently, normal growth of mammary glands of mice pregnant 18 days was determined using total DNA (desoxyribonucleic acid) as an index of growth(4). Levels of E.B. and P. which produce maximum mammary gland growth in ovariectomized rats(5) have been reported. The present study is concerned with levels of E.B. and P. which stimulate maximum growth of the mammary gland of male and ovariectomized female mice during period of 19 days.

Materials and Methods. Mature male and female Webster-Swiss mice were maintained on standard laboratory feed at constant temperature of 78±1°F. Ovariectomized mice received subcutaneous injections beginning day 14 postoperatively. Male mice were pretreated 28 days with 1.23 mg diethylstilbestrol per kg ground lab feed to insure good duct development before injection(6). E.B. and P. were dissolved in olive oil carrier at

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† Predoctoral Fellow of N.I.H.

TABLE I. Experimental Growth of Mammary Gland in Male and Female Mice.

Sex	Treatment	No. of mice	DFFT,* mg	DNA/mg of DFFT, μg	Total DNA, mg	Final mean body wt, g	Total DNA/100 g final body wt, mg
♀	Intact virgins	28	43.3	41.2 ± 1.6	1.76 ± .07[1]	29.5	5.93 ± .16
	33-day castrates	12	53.8	37.5 ± 2.9	1.95 ± .17	32.3	6.01 ± .30
	Sham-operated controls	7	45.7	33.6 ± 2.8	1.52 ± .10	27.3	5.69 ± .49
	E.B., 1 μg P., 1 mg	29	71.8	35.4 ± 1.2	2.59 ± .14[2]	30.6	8.46 ± .36
	E.B., 1 μg P., 2 mg	25	65.1	38.1 ± 1.0	2.55 ± .13[3]	30.2	8.48 ± .32
	E.B., 1 μg P., 3 mg	27	78.7	40.7 ± 1.1	3.20 ± .19[4]	32.4	9.73 ± .43
	E.B., 1 μg P., 4 mg	15	78.4	39.6 ± 1.6	3.15 ± .33	34.1	9.05 ± .62
	E.B., 1 μg P., 5 mg	16	67.9	38.9 ± 1.2	2.65 ± .22	31.4	8.64 ± .47
	18-days pregnant	28	123.5	52.0 ± 1.6	6.22 ± .37	37.4§	16.90 ± .69
♂†	Controls‡	20			1.20 ± .35[5]		
	E.B., 1 μg P., 1 mg	10	67.7	27.1 ± .9	1.82 ± .12[6]	34.6	5.27 ± .26
	E.B., 1 μg P., 2 mg	8	100.1	29.4 ± 2.0	3.12 ± .45[7]	39.6	7.70 ± .87
	E.B., 1 μg P., 3 mg	10	94.4	34.6 ± 1.6	3.10 ± .35[8]	38.3	8.17 ± .76
	E.B., 1 μg P., 4 mg	9	69.6	40.2 ± 1.7	2.77 ± .16[9]	39.9	7.02 ± .41

* DFFT, dry fat-free tissue.
† Pre-treated 28 days with 1.23 mg diethylstilbestrol/kg feed.
‡ From unpublished laboratory data.
§ Corrected for wt of fetuses.

Student's "t" probability: [1–2] .01; [5–6] .01; [4–2, 3] .05; [6–7, 8, 9] .01

concentrations of 1 μg E.B. and graded increments of P (1-5 mg) per 0.1 ml. Each animal received 0.1 ml carrier per day for 19 days. On day 20, animals were sacrificed and 7 mammary glands of each mouse were taken for DNA determinations as outlined by Brookreson and Turner(4). Remaining 3 glands were used for whole-mount observations.

Results. Statistical analysis showed experimental groups had total DNA levels significantly higher ($P<.01$) than appropriate controls (Table 1). Ovariectomized female groups receiving 1 μg E.B. and 3 mg P. were significantly higher ($P<.05$) in total DNA than groups receiving 1 or 2 mg P. No statistical differences were shown among female groups receiving 3 mg P. and more, while in male mice 2 mg to 4 mg P. showed no differences. However, when DNA was corrected for variations in body weight, 1 μg E.B. and 3 mg P. were optimal in males also.

Comparing control groups of intact virgin, ovariectomized, and sham-ovariectomized mice revealed castrates exhibiting higher levels of total DNA than virgin groups, while sham removal of ovaries resulted in lower DNA values than intact virgins. Neither of these differences was significant, however. On a total DNA basis, development of mammary glands in ovariectomized female group with highest DNA was 51.5% of 18-day pregnant controls and in male group 50.2%. When body weights were equalized, treated female group with highest DNA was 57.6% of 18-day pregnant controls and male group was 48.3%.

Whole-mount observations in both male and female mice indicated lobule-alveolar development in experimental groups most extensive in animals receiving 1 μg E.B. and 3 mg P.

Frequency distributions of DNA of virgin controls, ovariectomized females receiving 1

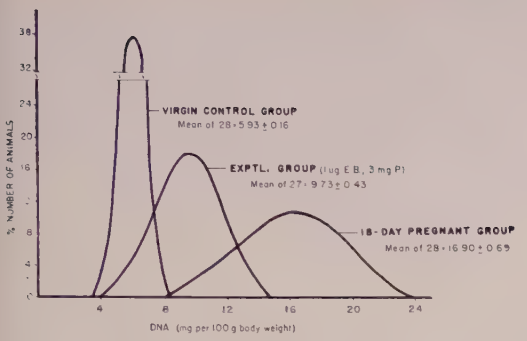

FIG. 1. Frequency distribution of DNA of mice mammary glands (7 of 10 glands). Pregnant mice on day 18 show mean DNA of 16.9 mg/100 g body wt with range from 8 to 24. Optimal level of EB and P stimulated mean DNA of 9.75 mg with range of 4 to 15 or only 57.6% of pregnant mice.

μg E.B. and 3 mg P. for 19 days, and pregnant group were not significantly different from normal distribution as shown by chi-square test (Fig. 1).

Discussion. Previous studies in rats(5) indicated that a level of 1 μg E.B. and 2 mg P. for 19 days developed mammary glands of ovariectomized females comparable to those of pregnant controls; levels of P. above 2 mg did not enhance or inhibit further growth. The present study indicates 1 μg E.B. and 3 mg P. for 19 days is optimal in mice. Since 2 mg P. and 5 mg P. did not produce as much growth, ratio of E.B. and P. in mice appears to be more critical in mice than in rats. In contrast to results in rats, experimental growth comparable to pregnancy was not reached in this study. Total DNA values of 3.20 $\pm$ 0.19 mg and 3.10 $\pm$ 0.35 mg for ovariectomized females and pre-treated males, respectively, were highest of experimental groups compared to 6.22 $\pm$ 0.37 mg for 18-day pregnant controls. DNA values per 100 g body weight were 9.73 $\pm$ 0.43 mg and 8.17 $\pm$ 0.76 mg for females and males, respectively, while pregnant controls reached 16.90 $\pm$ 0.69 mg. Thus, in experimental animals, mammary gland growth per unit of body weight in females was 57.6% and in males 48.3% of pregnant controls.

In comparing mammary growth of rats with mice, total DNA per 100 g body weight may be used. Moon *et al.*(5) reported a value of 7.63 $\pm$ 0.40 mg DNA per 100 g body weight. This includes 6 posterior glands only. Present study using 4 posterior and 3 anterior glands in mice shows a value of 16.90 $\pm$ 0.69 mg DNA per 100 g body weight. If each gland has approximately the same amount of DNA, then total DNA per 100 g would be 15.26 mg in rats and 24.15 mg in mice. Relatively larger mammary glands plus higher metabolic rate partially explain higher requirements of hormone per unit of body weight in mice compared to rats.

Experiments with E.B. and anterior pituitary extracts (APE)(7) in mice indicated that mammary gland growth comparable to pregnancy could be attained with 1 μg E.B., 3 mg P. and 0.4 mg APE. Such evidence leads to several explanations for inability of E.B. and P. to develop glands equal to pregnancy: (1) E.B. and P. in amounts used may not be stimulating adequate production and release of hormones from anterior pituitary; (2) placenta may play a role in mammary growth of mice while not doing so in rats. Some evidence to support the latter view has been presented(8,9,10). Placental factor may be similar to anterior pituitary hormone(s) or it may be a stimulant on AP synthesis and release. The third possibility for inability of experimental growth to equal that of pregnancy is greater sensitivity of mice to E.B. and P. ratio changes during normal pregnancy. It is known that ratios change throughout pregnancy. Various ratio changes have been attempted without success to date, however.

Summary. 1. Maximum growth of the lobule-alveolar system of mammary gland, as measured by total DNA, in ovariectomized female and estrogen-primed male mice (to develop duct system) was observed when 1 μg estradiol benzoate and 3 mg progesterone were administered daily for 19 days. (2) Maximum total DNA experimentally stimulated in female mice, on an equal weight basis, was only 57.6% of the DNA observed in normal mice pregnant 18 days. (3) Corresponding value for males was 48.3%. (4) These data suggest that a hormone of placental origin may be involved in mammary gland growth other than ovarian hormones.

(5) On an equal body weight and gland basis, the mammary glands of mice at end of pregnancy contain approximately 150% as much DNA as do rats.

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Active Immunization of Mice Against *Candida albicans.** (26405)

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Despite scanty unequivocal evidence, the possible beneficial role of immunity in human candidiasis has been suggested by clinical experience (*e.g.*, 1, 2) and in one case of pulmonary candidiasis, the use of rabbit antiserum was reported to be efficacious(3). Experimentally, immunologic studies with *C. albicans* have been largely confined to rabbits. Hurd and Drake(4) gave such animals repeated intravenous injections of heat-killed suspensions of a strain described as highly pathogenic for them. When agglutinin titers reached 1:2560 the vaccinated animals, as well as a control group, were given a dose of *C. albicans* defined as "one ml of sufficient concentration as to bring death on the seventh day." The authors concluded that the vaccination was not only ineffective but possibly harmful. Winner(5) found that normal rabbits possessed a high level of natural agglutinins but that actively or passively induced agglutinins did not protect the animals from an inoculum of *C. albicans* lethal for controls.

Since little immunologic work with *C. albicans* in mice was encountered in the literature and since with mice the use of larger groups would be facilitated, this problem was investigated by challenge of such animals which had been previously vaccinated with variously prepared materials, including sonically ruptured cells.

Materials and methods. *C. albicans*, strain 266, of human origin was used throughout this study. The culture was maintained on slants consisting of 1% casamino acids, 1% yeast extract, 2% glucose and 2% agar (CYE agar). Growth less than a week old was used to inoculate 150 ml of CYE broth in 500 ml Erlenmeyer flasks which were incubated on a rotary shaker at 37°C for 24-36 hours. The concentration of yeast particles was first estimated by direct microscopic counts using a hemacytometer and the viables were determined by the pour plate method.

Cells of *C. albicans* were washed and resuspended in saline so as to contain 2×10^8 particles per ml. From this stock, 3 different vaccines were prepared: (a) viable cells; (b) cells killed by exposure to merthiolate for 48 hours, then rewashed with saline; (c) cells killed by subjecting them to sonic vibrations in a 10 kc Raytheon oscillator for nearly 7 hours, the temperature of which was controlled by chilling to approximately 6°C.

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† Postdoctoral research trainee in epidemiology and mycology.

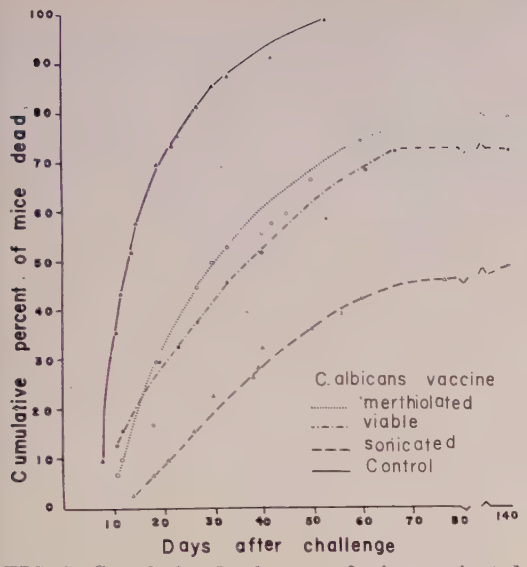

FIG. 1. Cumulative death rates of mice vaccinated with viable, merthiolate-killed or sonicated *Candida albicans* and challenged intrav. with 1×10^5 viable particles of the same strain.

Breakage of the cells was verified by direct microscopic examination, and complete loss of viability was determined by culture. All vaccines were of the same initial cellular concentration.

A preliminary virulence titration of the challenging strain in mice permitted selection of suitable doses for challenge, *viz.*, which would not overwhelm subtle degrees of resistance yet not be of such low virulence that death could not be used as the end-point for comparison of effects.

Six-week-old, white, male mice, obtained from Taconic Farms, were used for the studies. Animals were divided into 4 groups of 80-100 mice each. The first 3 groups were immunized with the living, merthiolate-killed or the sonically-killed vaccine. The fourth group constituted the control which received saline.

The vaccines described above were administered as repeated subcutaneous inoculations, 0.2 ml per inoculum, as follows: 40, 30, 20, 12, 5 and 1 days before intravenous challenge with 10^4 or 10^5 viable particles. Non-vaccinated mice were challenged concurrently. Mice were observed daily for deaths over a period of 20 weeks. The animals dying during the experiment and those sacrificed at the end of observation period were autopsied, the gross pathology recorded, and smears and cultures made from the suspect organs, usually the kidneys.

Results. When mice were challenged with 10^5 viable particles, 100% of 49 non-vaccinated mice died whereas of those that received the vaccine of sonicated cells only 50% (15 of 30) succumbed. The total number of deaths among those inoculated with the merthiolate-killed or the living vaccines was also less than in the controls (73% of 30 and 77% of 39 mice, respectively). A comparison of all death rates is given in Fig. 1.

When animals were challenged with 10^4 viable particles, essentially the same relative degree of resistance was observed. A total of 62% (28 of 45) of the non-immunized mice died, but incidence of deaths among those vaccinated was as follows: animals which received sonicated cells, 17% (5 of 30); animals pre-treated with merthiolate-killed cells, 36% (11 of 30); animals pre-treated with living cells, 43% (17 of 40).

The autopsies of immunized as well as non-immunized animals that died during the experiments showed various kidney lesions that were indistinguishable from each other and contained viable *C. albicans*. In the surviving animals, sacrificed 20 weeks after challenge, kidneys showed unilateral but extensive abscesses which were sterile upon culture.

Discussion. The data presented in Fig. 1 demonstrate that vaccination of mice with either merthiolate-killed, sonically-ruptured, or living cells of *C. albicans* protected a significant fraction of the animals against subsequent challenge with a homologous strain. Furthermore, the protection conferred by the sonicated vaccine was greatest, as determined by the following computations. Since the difference in total percentages dead between merthiolated and living vaccines was not significant among any of the groups, the results obtained with these 2 vaccines were pooled and compared with mice given sonicated vaccine. For those mice challenged with 10^5

particles, the observed difference between the sonicated group and all other vaccinated animals was greater than the theoretical ($X^2 = 6.14$; theoretical X^2 1 df (.95) = 3.84). Similarly, comparison of those mice challenged with 10^4 particles, showed the difference between those vaccinated with sonicated material and with the other types of vaccine, to have X^2 of 5.2, again greater than the theoretical, with the same confidence limits. Although complete protection was not attained, there can be little doubt that at least some degree of resistance was demonstrated. Whether or not this resistance was related to the presence of circulating antibody is being investigated.

Our conclusions therefore differ from those of Hurd and Drake(4) and of Winner(5) who, however, not only employed a different species of experimental animal but also possibly challenged with excessive doses inasmuch as both vaccinated and non-vaccinated animals died in less than a week.

Summary. Mice were vaccinated by repeated subcutaneous inoculations of living, merthiolate-killed, or sonically-ruptured cells of *C. albicans* and were then challenged intravenously with 10^4 or 10^5 viable particles of a homologous strain. Although not all of the vaccinated mice survived challenge, a significant degree of resistance was conferred since fewer deaths occurred among vaccinated animals.

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Runt Disease in Adult Tolerant Mice Induced by Intravenous Injection of Immunologically Competent Cells.* (26406)

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It is now well established that mice, rats, chicks, and other species, injected at birth or prior to birth with immunologically competent lymphoid cells from adult animals, often fail to grow, and succumb within a few weeks. This syndrome, which has been variously called runt disease, homologous disease, immunological disease, or wasting disease, has been found to be the result of an immune reaction elicited in the transferred, immunologically competent, adult cells by the antigens of the host. A similar phenomenon has been produced in adult F_1 hybrid mice injected with mature parent cells and in mice receiving a lethal dose of x-rays followed by administration of bone marrow cells taken from unrelated donors (For review see 1.) Finally, Casterman(2) recently described a similar type of syndrome in adult mice treated with large numbers of homologous lymphoid cells. In all of these situations, the clinical and gross pathologic findings have been similar, consisting of initial enlargement of the spleen, failure to grow, development of diarrhea, and terminating, often with infections, in a state characterized by extreme atrophy of the lymphoid tissues, especially the spleen and lymph nodes.

According to Billingham(1), the ability of the injected cells to induce this disease has 3 bases: 1) maturity and immunological competence of the inoculated cells; 2) presence in the host of transplantation antigens not present in the grafted cells; and 3) accep-

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TABLE I. Homologous Disease in Tolerant Mice Induced by Intravenous Injection of Spleen Cells.

Group	No. of mice	Mean body wt, g		Mean spleen wt, mg	Spleen wt, mg/100 g body wt
		Initial	Final		
Z normal	8	22.40	26.70	156 ± 21	584
Z tolerant of A*	8	25.05	27.00	169 ± 18	629
Z tolerant of A inj. with A cells†	14	22.40	21.90	273 ± 39	1246

* Tolerance was induced by intrav. inj. of A cells at birth.
† 20 million A spleen cells once a week for three weeks.

tance of the transplanted cells by the recipient without destructive reaction against these cells.

In the experiments herein described, attempts were made to induce immunological "runt disease" in adult mice tolerant of another homologous strain. The essential manipulation was injection of large numbers of immunologically competent cells derived from donors of the same strain as those used to induce tolerance at an earlier date. Specifically, an effort was made to produce runt disease in adult Z§ mice, previously made tolerant by injection of A strain spleen cells at birth, by further administration during adult life of large numbers of immunologically competent spleen cells from A strain donors. This paper reports the production of severe immunological disease using this technic.

Method. Mice of the Z and A strains were used. Z strain mice were injected intravenously at birth with a viable spleen cell suspension taken from adult donors of the A strain. Spleen cell suspensions were prepared by the method described previously (3) and the injection performed as recommended by Billingham and Brent (4). Approximately 40 days following spleen injection, treated animals received a full-thickness abdominal skin homograft taken from adult donors of the A strain. The technic of skin grafting was the same as that described earlier (3). After a period of observation ranging from 2 to 5 months, mice showing tolerance of A tissue, as revealed by successful establishment of the homologous skin graft, became the experimental group. These mice were perfectly well, without overt signs of homologous disease. They were then subjected to 3 weekly intravenous injections of approximately 20 million viable spleen cells from adult, male, A strain donors. Body weights were determined before the first injection and at weekly intervals thereafter. At the end of the fourth week, the animals were sacrificed, the spleen removed and weighed in a torsion balance. Spleen and lymph nodes were then fixed in 10% formaldehyde and embedded in paraffin, and histological sections were prepared and stained with hematoxylin and eosin in the usual manner.

Two groups of control animals, consisting of Z mice made tolerant of A tissue but not treated further, and normal, untreated mice of the same strain, were also weighed at weekly intervals and sacrificed at the same time as the group of experimental mice. Histological studies of spleen and lymph nodes were also carried out in the control mice.

Results. The results are summarized in Table I. The normal, untreated Z strain mice gained an average of almost 4 g in body weight during the 4 weeks of experiment. In this group the mean absolute weight of the spleen at the end of experiment was 156 ± 21 mg. The control group of Z mice previously made tolerant of A tissue gained almost 2 g in the same period, and mean weight of the spleens was 169 ± 18 mg. The difference between average spleen weights in these 2 control groups is not statistically significant. Finally, the experimental group of Z strain mice, tolerant of A strain tissue and injected with additional spleen cells from strain A donors, showed loss of body weight

§ Z is used to designate mice of C3H strain originally obtained from Dr. J. J. Bittner's mouse colony.

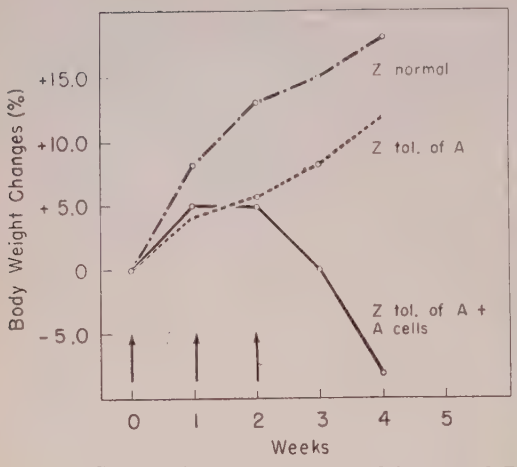

FIG. 1. Changes in body wt observed in normal Z strain mice, Z tolerant of A tissue and Z tolerant of A tissue inj. with mature competent A strain spleen cells. Arrows indicate time at which injections were performed in the latter group.

and a marked increase in weight of spleen. Spleens of the animals of this group weighed on the average 273 ± 39 mg. The difference between mean spleen weight of this group and that of either the normal or tolerant control groups is significant at the 5% level.

Fig. 1 illustrates changes in body weight observed during the experiment in the 3 groups of mice involved. While both groups of controls, *i.e.*, normal Z strain mice and Z mice tolerant of A tissue, gained in body weight during 4 weeks of observation, the experimental group of tolerant mice injected with additional mature spleen cells showed an initial increase during the first week, followed by a leveling-off after the second injection, and a marked decrease in weight beginning about the time of the third injection of homologous spleen cells.

Fig. 2 illustrates pathologic changes observed in the spleens of representative animals of each group sacrificed at the end of experiment. The lymphoid follicles in the spleens of the normal mice, as well as those in the tolerant, non-runted animals, are well preserved. In contrast, sections from tolerant animals which had subsequently been treated with large numbers of adult spleen cells from strain A donors, showed a complete disorganization of the splenic tissue, with less of the normal architecture, apparent necrotizing changes, and complete disappearance of lymphoid follicles. Similar atrophic changes were also observed in the inguinal and axillary lymph nodes removed from the runted animals, although the alter-

FIG. 2. Photomicrographs of spleen sections taken from normal and experimental animals. Hematoxylin-Eosin. (A) Normal Z mouse; (B) Z tolerant of A tissue; (C) Z tolerant of A tissue inj. with immunologically competent A strain cells. Magnification 154 ×.

ations observed were less profound than those seen in the spleens of these mice.

Discussion. The results of these experiments demonstrate that it is possible to induce runt disease in adult Z mice if these animals have previously been made tolerant of A tissue homografts. The effective procedure was simply injection of an additional complement of homologous cells. In these animals, the tolerant state had been induced at birth by a single injection of approximately 4 million mature, A strain spleen cells per mouse. This number of homologous spleen cells was sufficient to produce tolerance but apparently insufficient to produce runt disease in the newborn animals in this donor-host combination. When these animals had reached 2 to 5 months of age, runt disease was reproducibly produced by giving the tolerant recipients 3 weekly injections of spleen cells from the strain of mice to which they possessed tolerance.

The syndrome observed appeared to be similar to the "graft versus host" reaction obtained in: 1) newborn animals injected with homologous lymphoid cells; 2) F_1 hybrid mice receiving inoculation of mature lymphoid cells from donors of either parental strain; and 3) mice rescued from lethal irradiation by an inoculation of homologous bone marrow cells. Many mice treated in this way died of their homologous disease. This lethality as well as the pathologic findings, particularly atrophy of the lymphoid tissues and loss of body weight, are indications that the underlying mechanisms might be similar to that observed in newborn or adult animals accepting homologous lymphoid cells. Animals made tolerant by injection of homologous cells at birth are incapable of reacting against these cells and are, therefore, defenseless when invaded by immunologically competent cells having the full capacity of reaction against the host antigens.

It is interesting to note that Z strain mice tolerant of A tissue which are not given further treatment do not show any striking clinical or pathologic disease. In this donor-host combination, immunological runt disease is indeed induced in many Z animals injected with adult A cells at birth. However, the animals surviving this pretreatment grow and develop in a normal fashion, and at the time studied have lymphoid tissue of normal structure. Dosage of spleen cells given at birth is doubtless an important factor in the outcome of these animals. Their clinical and histologic well-being may be explained by assuming that the cells injected during the neonatal period have themselves gradually become tolerant of the antigens of the host and hence are incapable of reacting against these antigens, by a mechanism such as the exhaustive sensitization of the grafted cells, proposed by Simonsen(5) or the development of tolerance of adult cells by some other mechanism. Certainly the development of tolerance by adult cells is implied in our previous studies with tolerance induced in adult mice by parabiosis or intravenous injection (6,7).

When these doubly tolerant animals are then treated with an additional complement of homologous, immunologically competent cells, these new cells, if provided in sufficiently large numbers, attack the host's tissues, particularly the lymphoid tissues, and accomplish the destruction of the recipient. These observations might then be interpreted as evidence in favor of development of tolerance or exhaustion of the adult cells injected in the neonatal period.

Further, the large number of immunologically competent homologous cells might be able to cope with the antigens available in the tolerant recipient and only experience what Simonsen termed productive sensitization. Since productive sensitivity of the grafted cells is considered by Simonsen to be a precondition of exhaustion, one wonders whether the larger number of cells used to produce runt disease in our experiment would cause runting in tolerant animals if given in small divided dosages during a longer period of time. Experiments to answer this question are in progress.

Perhaps the greatest significance of these observations, besides their theoretical interest, is that they provide a model for production of immunological-homologous disease in

adult animals in which it will be possible to study the impact of the "graft versus host" reaction in absence of such complicating factors as neonatal immunological incompetence, neonatal failure of gamma globulin and protein synthesis, rapid growth and development, shifting blood and tissue volume, or of the devastating general effects of irradiation. Investigations of the effect of this "graft versus host" reaction on protein synthesis, immunological competence of the host, and the effect of prior immunological commitment of administered cells on capacity to develop the reaction, may now be effectively investigated.

Summary. 1. Runt disease in adult mice of the Z (C3H) strain made previously tolerant of A tissue by antigenic exposure at birth was obtained by injecting these animals intravenously with large numbers of immunological competent spleen cells taken from A strain donors. 2. The main pathological findings in these animals were: a) marked body weight loss; b) Splenomegally and c) Atrophy of lymphoid tissue followed by death.

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Anaerobic Conversion of Acetate to Squalene by Rat Skin.* (26407)

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When rodent skin is incubated with labeled acetate *in vitro,* sterol synthesis is substantial (1) and much of the label is found in sterols other than cholesterol(2). The relative activities of these sterols cannot however, be taken as a measure of precursor-product relationships since skin is so complex anatomically, and rate of penetration of acetate into different parts of the skin probably varies. Grinding of the skin has thus far yielded inactive preparations. Another approach to the problem might be possible if an active sterol precursor could be established within the tissue without the sterols themselves becoming labeled. The present studies describe conditions for accumulation of labeled squalene in rat skin.

Methods. In each experiment, one gram of rat skin was cut into pieces of 10 sq mm and incubated for 3 hours at 37°C in 6 ml of saline-phosphate buffer which contained 2.0 μc/0.4 μM of acetate-1-C^{14}. The buffer (pH 7.4) was prepared by mixing equal volumes of isotonic saline and 0.1 M sodium phosphate solutions. Potassium chloride and magnesium sulfate were added to final concentrations of 0.0125 M and 0.0026 M respectively. Nitrogen was bubbled into the solution for 10 minutes; all incubations were carried out under nitrogen. At the end of incubation period, the pieces of skin were filtered from the medium and each gram was saponified for 4 hours under nitrogen in 10 ml of 50% aqueous ethanol (v/v) which contained 20% of KOH (w/v) and 7.5% of pyrogallic acid (w/v). The unsaponified fraction was ex-

* The initial experiments are recorded in detail in a thesis entitled Biosynthesis of Sterols as Altered by Carcinogens or Nicotinic Acid, Univ. of Wisconsin, 1960. Supported in part by grant from Nat. Inst. of Health to C. A. Baumann, and by funds available through State Univ. of New York.

TABLE I. Effect of Glucose and Insulin on Incorporation of Acetate into Squalene and Sterols by Rat Skin.*

	Additions				Activities	
	Glucose, M	Insulin, u/flask	Ferricyanide, M	Atmosphere	Squalene, cpm/g skin	Sterols,† cpm/g skin
1.	—	—	—	O_2	301	973
2.	—	—	—	N_2	186	3
3.	.005	—	—	"	443	—
4.	.017	—	—	"	618	—
5.	.05	—	—	"	768	21
6.	.17	—	—	"	622	—
7.	—	1.0	—	"	188	—
8.	.05	.25	—	"	803	—
9.	.05	1.0	—	"	858	12
10.	.05	2.0	—	"	862	—
11.	.05	—	.0075	"	1090	32
12.	.05	1.0	.0075	"	1620	31
13.	.025	—	—	O_2	492	1370

* Each flask contained 1 g of skin, 6 ml of saline-phosphate buffer, 2 μc/0.4 μM of acetate-1-C^{14} at pH 7.4. All incubations were for 3 hr at 37°C. Each value represents the avg of at least 8 samples from 3 separate incubations.

† Precipitated as the digitonides.

tracted into pentane which was washed, dried and concentrated under nitrogen. The fraction containing squalene was obtained by chromatography on aluminum oxide(3). Purification of the labeled sterols and assays of sterol and radioactivity have been described (4).

The squalene content of the first chromatographic fraction was estimated as follows: samples from whole skin (7750 cpm) or from "dermis" (3350 cpm)† were diluted to 50 mg with unlabeled squalene and reacted with dry HCl. The hexahydrochlorides of squalene were recrystallized from a mixture of absolute methanol and chloroform until the mother liquor and precipitate reached the same constant specific activity calculated as squalene. The specific activity of 4 samples of squalene from whole skin ranged from 117 to 135 cpm/mg and that from the "dermis" was between 57.3 and 62.8 cpm/mg. At least 75.5 to 87.2% $\left(\frac{\text{Sp. Act. final} \times 100}{\text{Sp. Act. initial}}\right)$ of the activity isolated in the first chromatographic fraction of whole skin extract was squalene; 85.5 to 93.7% of the activity isolated in the first fraction of "dermis" was squalene. No change in activity was observed when the crude fractions were reacted with thiourea.

Experimental. When rat skin was incubated with labeled acetate under nitrogen, the incorporation of label into the unsaponified fraction (sterol and squalene) was only 14% of that observed under aerobic conditions. Sterol synthesis virtually ceased under anaerobic conditions, and instead, labeled squalene accumulated (lines 1 and 2, Table I). This is in harmony with the finding that liver homogenates require oxygen for conversion of squalene to lanosterol(7). When glucose was added to the medium at concentrations of 0.005 to 0.17 M, a marked increase in squalene synthesis took place (Table 1, lines 3 to 6). Lower concentrations failed to affect squalene synthesis. Boiled liver filtrate (equivalent to 0.5 g liver/flask), glucose-6-phosphate (0.005 M), succinate (10^{-4}M),α–ketoglutarate (10^{-4}M) or ATP, DPN or TPN (1 mg each/flask) produced little or no stimulation.

Insulin (U-40, E. R. Squibb & Sons) enhanced the uptake of acetate into squalene in presence of glucose (lines 7 to 10, Table I). Amounts less than 0.25 units/flask were less active and 1 to 2 units/flask produced consistently higher results. In the presence of both ferricyanide and glucose, the effect of added insulin was even greater (lines 11 and 12, Table I). Upon addition of insulin, ac-

† The epidermal mucosa (0.3 to 0.5 mm) was removed by a single horizontal section made prior to incubation. The dermis in this case contained essentially all of the sebaceous glands (5,6).

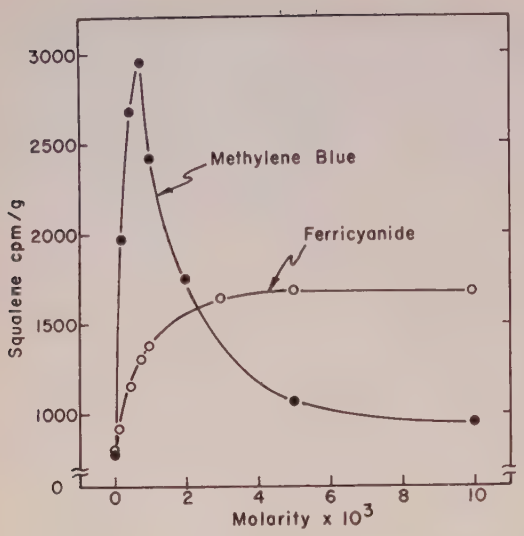

FIG. 1. Effect of methylene blue and ferricyanide on anaerobic incorporation of acetate into squalene. Each flask contained 6 ml of buffer, glucose (0.05 M), 2 units of insulin, 2 μc/0.4 μM of acetate-1-C^{14} and 1 g of skin and was adjusted to pH 7.4 after addition of the electron acceptor. Although there was no quantitative determination of extent of dye reduction, all of the methylene blue was in the leuco form when concentration was less than 10^{-3}M. Methylene blue was added as its chloride; ferricyanide as $K_3Fe(CN)_6$. Additions of potassium ion (KCl) in absence of ferricyanide produced no change.

tivity in the sterol fraction was increased by only 28 cpm/g skin.

When the buffer contained both glucose and insulin, addition of 10^{-3}M methylene blue produced a 2.5-fold increase in the activity of squalene (Fig. 1). However, with greater concentrations of methylene blue, the activity dropped off rapidly. Additions of ferricyanide to the same medium only doubled the incorporation over a rather wide range of concentrations.

In some experiments, after 3 hours of incubation under nitrogen, the pieces of skin were filtered from the anaerobic medium, washed with saline and resuspended in freshly-oxygenated, saline-phosphate buffer containing 27 mg of glucose and 4μM of unlabeled sodium acetate in each flask. The skin was then incubated for 5, 12, 60 and 90 minuates under oxygen. The squalene labeled *in situ* disappeared rapidly and activity accumulated in the sterol fraction (Table II). After 90 minutes of aerobic incubation 88% of the activity which was lost from the squalene fraction was recovered in the sterol fraction. As much activity was converted from squalene to sterol in 90 min as previously observed for 3 hour experiments with labeled acetate (lines 1 and 13, Table I; Table II). In subsequent experiments, significant amounts of labeled sterols were found after 2 minutes of aerobic incubation. Although the activity incorporated into the squalene fraction under nitrogen was greater when methylene blue was the electron acceptor, only a few counts were transferred to the sterols during aerobic incubation.

Conclusions. Others have observed the stimulation produced by glucose(8), insulin (9) and electron acceptors(6,10) on lipogenesis in various tissues under oxygen. The present data show that a combination of these materials enhanced the anaerobic conversion of acetate to squalene in pieces of rat skin. The amount of activity incorporated (1620 cpm/g skin) was sufficient to serve as labeled squalene for subsequent conversions of squalene into sterol precursors or the sterols themselves.

In contrast to human skin, rat skin contains relatively little squalene(11), and most of the activity incorporated into the unsaponified fraction of rat skin under oxygen is associated with the sterols rather than with squalene. Aerobic incubation of human skin with acetate, on the other hand, yields substantial amounts of labeled squalene(5,12). Presumably, the yield of labeled squalene by human skin could be increased by an anaerobic incubation in fortified buffers like those used in the present study.

TABLE II. Conversion of Squalene to Sterols by Rat Skin.

Length of incubation, min.	Activities		
	Squalene	Sterols	△ Squalene
	—cpm/g skin—		
0	1730	43	—
5	1380	221	350
12	677	390	1050
60	474	853	1260
90	403	1160	1330

The skin was previously incubated for 3 hr under nitrogen. During the aerobic part, each flask contained 27 mg glucose and 4 μM unlabeled acetate.

Summary. 1. Rat skin incubated anaerobically accumulated labeled squalene from acetate and only negligible amounts of labeled sterols. Incorporation of acetate into squalene was enhanced markedly by the presence of glucose in the medium. 2. Insulin, ferricyanide and methylene blue enhanced squalene synthesis *in vitro* in presence of glucose. Increasing amounts of insulin or ferricyanide produced regular increases in squalene synthesis until a plateau was reached; methylene blue on the other hand showed a sharp maximum at 10^{-3}M, with a decline at higher concentrations. 3. Skin containing labeled squalene accumulated labeled sterol on subsequent incubation under oxygen.

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Activity of Pituitary-Adrenal Cortex Axis During Acute and Chronic Reserpine Treatment.* (26408)

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It has been demonstrated that reserpine treatment affects endocrine systems(1,2,3). It was reported that reserpine interferes with the estrous cycle in mice and rats(1,4), ovulation and menstruation in monkeys(5), prolactin production and secretion in rats, rabbits and women(6,7,8), and normal function of the pituitary-gonadal axis(9). Reserpine was found also to effect recession of exophthalmus(10), thyroid inhibition(11), and to have antithyroid activity(12).

It has been suggested that adrenal hypertrophy and ACTH hypersecretion occur during reserpine administration(1,13,14,15,16). On the other hand, inhibition of pituitary ACTH release after administration of reserpine(17,18), and suppression of adrenal hypertrophy in male mice(19) have been reported.

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In view of these conflicting reports we have studied the mode of action of reserpine treatment on the pituitary-adrenal cortex axis.

Materials and Methods. Reserpine stock solution was prepared according to the method described in Martindale's Extrapharmacopoea(1959), and diluted to the required concentration. Studies were carried out on adult male guinea pigs, adult male rats, and on human patients.

Exp. 1. Five adult guinea pigs (600± 30 g) were injected subcutaneously with 0.2 mg/kg reserpine daily for 7 days. The animals were then sacrificed, the adrenals weighed and histological sections prepared and studied.

Exp. 2. Ten adult male rats (200 ± 20 g) were injected subcutaneously with 0.01 mg/kg reserpine daily for 10 and 40 days respectively while 5 animals served as controls.

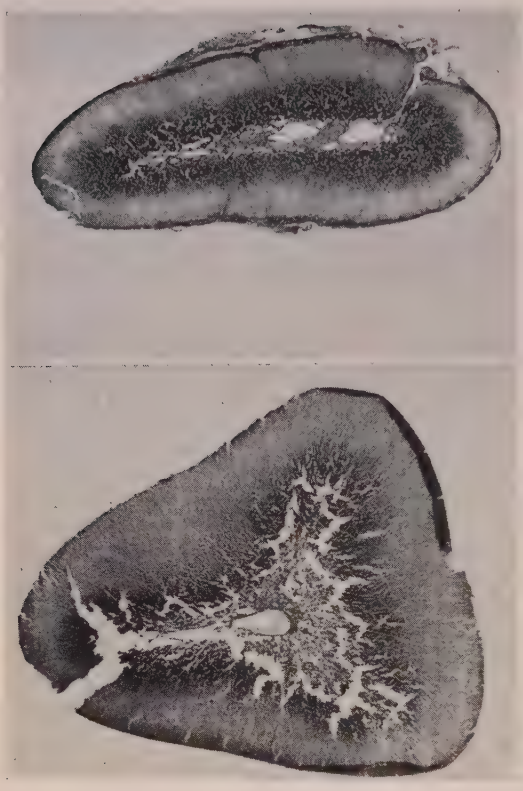

FIG. 1. Histological sections of left side adrenals of guinea pigs (10 ×). Top: control, 165 mg. Bottom: reserpine treated, 255 mg.

The animals were sacrificed and the adrenals dissected and weighed.

Exp. 3. Ascorbic acid depletion was determined by the method of Roe(20) on adrenals of 15 adult male rats (250 ± 25 g) treated with 0.2 mg/kg reserpine daily for 1, 2 and 7 days respectively, one hour after last administration.

Exp. 4. 17-KS and 17-OHCS were determined periodically by the method of Sulman (21,22) on 24 hr urines of 7 human patients receiving 1 mg reserpine daily for 30-40 days.

Results. Administration of reserpine to guinea pigs (Exp. 1) for 7 days caused adrenal hypertrophy of 60-80% by weight. The histological sections showed an enlarged adrenal cortex, especially in the *zona fasciculata* (Fig. 1). Adult male rats treated with reserpine (Exp. 2) were found to have significantly hypertrophied adrenals on the 10th day of treatment, ranging between 30-50%. Forty days' treatment resulted in almost no hypertrophy (Table I). A higher adrenal ascorbic acid depletion (Exp. 3) was found after the first injection than in chronic treatment at the same dose level (Table II). The 17-KS content in 24 hr urines of 7 human patients (Exp. 4) showed higher titers during the first 7-10 days (average 17 mg/d), then tended to return to normal (average 12 mg/d) by the 30-40th day. Corresponding average values for 17-OHCS excretion were 5.5 mg/d at the beginning, declining to 3 mg/d with continued treatment.

Discussion. Hertting *et al.*(13) found that adrenal hypertrophy in rats treated with reserpine could be inhibited by simultaneous administration of cortisone, and suggested that the hypersecretion of ACTH caused by reserpine had been inhibited by cortisone. Tindal (14) reported thymus atrophy in rabbits, indicating pituitary-adrenal cortex hyperactivity during reserpine administration. Higher 17-OHCS blood levels in monkeys were reported after i.v. injection of 1 mg/kg reserpine(15), estimated to be equivalent to the elevation caused by 16 mg/kg exogenous ACTH. Similar results were noted in man(16).

While our results showed that reserpine has a stimulating effect on the pituitary-adrenal cortex through acute treatment, as revealed by adrenal hypertrophy, adrenal ascorbic acid depletion and increased 17-KS

TABLE I. Adrenal Weight of Reserpine Treated Adult Male Rats at a Dose Level of .01 mg/kg.

No. of rats	Days of treatment	Wt of adrenal, mg
5	0 (control)	27 ± 1.5*
5	10	35 ± 2.1
5	40	28 ± 3.2

* ± stand. errors.

TABLE II. Ascorbic Acid Depletion in Adrenals of Adult Male Rats Treated with .20 mg/kg Reserpine Daily.

No. of rats	Days of treatment	Ascorbic acid contents, mg/100 g adrenal
5	0 (control)	310 ± 21*
5	1	170 ± 17
5	2	210 ± 15
5	7	260 ± 24

* ± stand. errors.

and 17-OHCS levels, they indicate that this effect is transitory. These findings suggest that reserpine is not a specific stimulant of the hypophysis which causes hypersecretion of ACTH, but that the acute ACTH hypersecretion following reserpine administration may be due to the non-specific transitory stress reaction brought about by the drug.

Summary. Acute reserpine treatment of guinea pigs, rats and human patients resulted in activation of the pituitary-adrenal cortex axis demonstrated by hypertrophy of adrenals, adrenal ascorbic acid depletion and higher 17-KS and 17-OHCS levels. These changes disappeared during chronic treatment. It is hence suggested that reserpine through its transistory stimulating effect on the pituitary-adrenal cortex axis acts as a non-specific stimulus, which loses its effect on chronic administration.

Our thanks are due to Mr. E. Zawojski for devoted technical assistance.

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Propagation of Measles Virus in Suspensions of Human and Monkey Leucocytes.* (26409)

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Many viral diseases are associated with a reduction of circulating leucocytes which as a rule is most conspicuous when patients manifest clinical and laboratory evidence of diffuse viral dissemination. The leucopenia accompanying measles is characterized by an absolute reduction of both lymphocytes and polymorphonuclear leucocytes. In attempting to elucidate factors responsible for leucopenia in this disease experiments were designed to determine whether *in vitro* leucocytes of several animal species including man were capable of supporting the growth of measles virus.

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† Postdoctoral Research Fellow, U.S.P.H.S.

Materials and Methods. Preparation and

maintenance of leucocyte suspensions. Fresh venous blood was withdrawn aseptically from healthy human subjects, leukemic patients, a cynomolgus monkey, a domestic rabbit, mongrel dogs and chickens. Fresh cardiac blood was obtained from mice (Webster strain) and guinea pigs. To each 10 ml of blood was added 0.0025 mg of sterile heparin and 2.5 mg of sterile Bacto-Phytohemagglutinin (Difco). Samples were shaken gently by hand for 3-10 minutes and then centrifuged at 300-500 rpm for 3-5 min. at room temperature. Supernatant fluid and buffy coat, containing some red blood cells and the majority of leucocytes were removed and washed 3 times in medium 199(1) and finally resuspended in medium 199 containing 20% calf serum, 50 units of penicillin/ml and 50 μg of streptomycin/ml. When serum of human subjects or animals had been found to be free of measles neutralizing antibodies, heparinized whole blood was usually employed without further treatment as a culture system. Nucleated cell counts of final preparations varied between 5×10^6 and 10×10^6 cells/ml. Counts were not performed on chicken blood. Aliquots of 1 ml of washed or unwashed blood cell suspensions were delivered to 150 x 16mm test tubes. *Virus.* Three stocks of the Edmonston strain of measles virus were used(2). The first had been passed serially 29 times in human kidney cell cultures and the second 28 times in human kidney cell cultures and once in dog kidney cell culture. The third consisted of the 14th chick embryo cell culture passage of the attenuated Edmonston strain(3). These stocks will be referred to, respectively, as HK virus, DK virus and attenuated virus. *Viral Assay.* Infectivity titers were determined in cultures of a continuous line of human amnion cells designated WS in this laboratory(5). Serial ten-fold dilutions of leucocyte suspensions previously inoculated with measles virus were prepared in cold bovine amniotic fluid medium and immediately 0.1 ml aliquots of each dilution were added to each of 3 WS cell cultures. Cultures were kept stationary at 37°C and observed for formation of typical giant cells on the 5th, 10th and 14th days.

WS cells were prepared and nourished in Eagle's basal medium(6,7) modified as follows: $MgSO_4$ (0.1 g/1) was substituted for $MgCl_2 \cdot 6H_2O$ (0.17 g/1), cystine was increased to 24.03 mg/1, methionine was decreased to 7.46 mg/1; Inositol (0.4 mg/1) (8) and supplemental arginine (17.42 mg/1) were added. Tissue culture fluids were changed on the 5th and 10th days after inoculation and cultures were discarded after 2 weeks.

Experimental. Multiplication of measles virus in human white cells was demonstrated in the following experiment. Each of 2 tubes containing 1 ml of washed human blood cell suspension was inoculated with 0.1 ml of a 10-fold dilution of stock HK virus and closed with solid stoppers (West Co., Phoenixville, Pa.—white, non-toxic). Undiluted stock HK virus had an infectivity titer of 3.8 log 10 TCD_{50}/0.1 ml. After these suspensions had incubated at 37°C for 4 days in a roller wheel turning at about 18 rph, 0.1 ml aliquots were passed to freshly prepared washed blood cell cultures. Third and fourth passages were made at 4 day intervals. Aliquots representing a dilution of the original stock HK virus of 10^5 times were taken from the final passage on the 4th, 7th and 14th days and titrated for infectivity. Titers were recorded respectively as 5.3, 4.8 and 1.8 log 10 TCD_{50}/0.1 ml.

In contrast, when tubes containing 1 ml of medium 199 plus 20% calf serum but no leucocytes were inoculated with 0.1 ml of undiluted HK virus and later assayed for infectivity, virus could not be demonstrated after 2 days incubation. Tubes containing human serum free of measles antibody were similarly inoculated. From these only 0.75 log 10 TCD_{50}/0.1 ml of HK virus was recovered after 2 days and none after 7 days. Suspensions of red blood cells and of leucocytes which had been rapidly frozen and thawed also failed to support survival or multiplication of measles virus.

In suspensions of both washed and unwashed leucocytes multiplication of virus was demonstrated during a single passage. In each experiment the pooled contents of 2 or

TABLE I. Maximal Infectivity Titers Found in Blood Cell Suspensions after Inoculation with Measles Virus.

Exp. No.	Source of blood cell suspensions	Virus	Titers (log 10 TCD_{50}/0.1 ml) of suspensions, days after inoculation* 0 day	4 days	7 days	14 days
1	Healthy human	HK	1.8	2.45	3.50	NA†
2	" "	"	1.8	1.75	3.50	NA
3	" "	"	1.8	4.75	4.75	0
4	" "	"	1.8	4.25	4.50	1.75
5	" "	"	1.8	3.50	4.25	0
6	" "	"	1.8	2.75	3.75	0
7	" "	"	2.8	3.00	4.25	2.5
8	" "	"	2.8	2.66	2.50	NA
9	" "	Attenuated	.8	2.25	2.75	0
10	" "	"	.8	3.25	2.50	0
11	Leukemic patient	HK	1.8	4.75	3.75	0
12	" "	"	1.8	2.75	2.75	NA
13	Cynomolgus monkey	"	1.8	3.25	3.50	0

* Titers of the stock HK and attenuated viruses were 3.8 and 2.8 log 10 TCD_{50}/0.1 ml. Zero day titer represents the calculated infectivity titer of the suspension immediately after inoculation with diluted stock virus. All titers were determined in WS cells.

† Not assayed.

more stock virus ampoules were employed as inocula and at least 2 blood cell suspensions were inoculated with 0.1 ml, appropriately diluted in bovine amniotic fluid medium and maintained at 37°C in a roller wheel. Aliquots of blood cell suspensions were pooled from 2 or more tubes and infectivity titers determined on the 4th, 7th and 14th days after inoculation. Early experiments showed that infectivity titers were maximal on the 4th to 7th days. Only rarely could virus be detected after 3 weeks. Results of these experiments are presented in Table I (Exp. 1-10).

Experiments were performed to determine whether in suspensions of human leukemic and certain animal leucocytes multiplication of measles virus occurred. Samples of blood were withdrawn from 2 children ill with acute leukemia. One child had a differential white cell count of 96% blast forms, 3% lymphocytes and 1% polymorphonuclear leucocytes. The other child's count showed 99% blast forms and 1% lymphocytes. The immature cells of both patients were considered likely to be lymphoblasts by the Tumor Therapy Service at The Children's Hospital Medical Center. HK virus, the only virus tested, multiplied in suspensions of wbc from these children (Table I, Exp. 11-12).

Attempts were made to propagate measles virus in leucocytes obtained from 6 different animal species. At least 2 separate specimens of blood were drawn from each animal. It was necessary to pool the cardiac blood of 6 mice for each experiment requiring the use of mouse blood. When mouse or chicken blood was used, the cells were not washed. In the case of all other animals both washed and unwashed blood cells were employed. Leucocytes obtained from chickens, dogs, rabbits, mice and guinea pigs did not support the multiplication or survival of HK virus. The single monkey used had been immunized with attenuated measles vaccine(8) and therefore all samples of its blood were washed. On 2 occasions HK virus failed to multiply in monkey leucocytes, but in one experiment propagation of the virus was demonstrated (Table I, Exp. 13). It was postulated that DK virus, having been adapted to dog kidney tissue culture, might multiply in canine leucocytes and that attenuated virus, having been adapted to chick embryo tissue culture, might multiply in chicken leucocytes. Canine suspensions were inoculated with 310 TCD_{50}/0.1 ml of attenuated virus, and avian suspensions with 63 TCD_{50}/0.1 ml of attenuated virus. In neither was evidence of multiplication obtained.

Preliminary observations have shown no significant difference in the rates of disappearance of particular cell types or the drop in total white cell counts in inoculated as

compared with uninoculated human leucocyte cultures. In both, virtually all polymorphonuclear leucocytes disappeared by the 4th day of incubation. Microscopic examination of infected and non-infected leucocytes stained with Wright's and acridine orange (9) stains failed to reveal distinctive morphologic variations. Efforts were made to localize the site of intracellular virus by means of the indirect fluorescent antibody technique (10). To date the presence of non-specific staining material has made it difficult to interpret the results.

Discussion. Occurrence of viremia in measles was first established in 1905 by transmission of the disease to susceptible subjects with whole blood(11) derived from patients. Measles virus has been isolated in tissue cultures from the blood of humans and monkeys in this laboratory(2,12). Papp transmitted the infection to children using suspensions of washed leucocytes obtained from measles patients(13). Peebles isolated the virus in tissue cultures from the buffy coat of typical cases(14). In this study we have shown that propagation of the virus is supported by suspensions of human and simian leucocytes. Taken as a whole these observations suggest that the virus during the viremic stage is closely associated with the blood and in particular with the blood leucocytes.

Earlier researches demonstrated that certain viruses of animal origin could be propagated in cultures of rabbit, porcine and avian leucocytes(15,16,17,18) after multiplication of the cells had taken place. Moreover, as we have now found with measles virus and primate cells, Dunne and his co-workers observed that Newcastle Disease virus multiplies in suspensions of porcine blood leucocytes. Although no direct evidence as yet has been obtained for multiplication *in vivo* of viruses in circulating leucocytes, these findings *in vitro* strongly suggest that it occurs in these elements. If so, as Dunne has postulated, one factor underlying the leucopenia can be visualized, since infection of the susceptible cell usually terminates in its destruction. Infection and death of immature members of the leucocytic series may, however, also play a significant role. Supporting data are not available in the case of measles and many other viral infections, but in feline panleucopenia(19) nearly all the cellular elements of the bone marrow appear to be attacked and destroyed by the responsible virus.

Our attempts to distinguish infected from uninfected cells morphologically and by means of immunofluorescence staining have so far proved unsuccessful. It therefore remains to be determined which of the leucocytic series are capable of supporting viral multiplication. Dunne and co-workers noted that in porcine cultures, polymorphonuclear leucocytes had essentially disappeared during the first 24 hours of incubation. Accordingly, they concluded that hog cholera virus multiplied in the remaining mononuclear cells. The data from our serial differential counts also suggest that measles virus reproduces itself in mononuclear cells. These data are supported by the growth of the agent in leukemic blood essentially devoid of polymorphonuclear cells. Moreover, inoculated normal white blood cell suspensions yielded the highest concentration of virus between the 4th and 7th days of incubation when a negligible number of polymorphonuclear leucocytes were present. Extracellular virus possibly derived from polymorphonuclear cells during the first 3 days of incubation would have been largely inactivated at this time. Thus, although viral multiplication appears to occur chiefly in mononuclear cells, the possibility that it also takes place to a certain extent in polymorphonuclear cells cannot at present be excluded.

The data now available do not indicate whether viral multiplication occurs in leucocytes originally present in the blood specimens or only in descendents of these cells. In view, however, of the rapidity of increase and decline of virus and the slow increase in mononuclear leucocytes observed in plasma roller tube cultures(18) it seems probable that the agent multiplies, at least in part, in surviving cells originally present in the circulating blood of the donor. Therefore, in this communication we have referred to the blood

cell preparations as "suspensions" rather than "cultures."

Summary. Multiplication of measles virus has been demonstrated in suspensions of human and simian blood leucocytes. Under the experimental conditions employed the virus failed to multiply in canine, rabbit, guinea pig, mouse and chicken blood leucocytes. Evidence is presented which indicates that multiplication takes place in mononuclear cells.

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Response of Rats of Various Ages to Erythropoietin.* (26410)

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New-born rats develop an anemia soon after birth and it becomes most severe when the animals are 15-20 days of age(1,2). In the rat this is the period of maximum growth rate of the body as a whole as well as the period of maximum growth rate of total circulating red cell volume. During the time that the anemia is developing, iron kinetics studies reveal that erythropoiesis is proceeding more rapidly than at any other time in the life span of the animal(3). From these observations it has been suggested that the anemia in the rat results from the disproportionate growth of the body in relation to the ability of the marrow to supply erythrocytes. This implies that the marrow is functioning normally and at maximum capacity during the anemic period. It has been shown that rats do not show an erythropoietic response to hypoxia during the neonatal period(4).

It was considered of interest to determine whether erythropoietin, which is active in adult rats, would stimulate erythropoiesis during the period of neonatal anemia.

Materials and methods. Both total circulating red cell volume measurements and Fe^{59} red cell incorporation studies were used to judge the erythropoietic response to erythropoietin. Groups of normal male rats of the Long-Evans strain of 12, 23, 87, 230, and 470 days of age were used to determine the effect of erythropoietin on total circulating red cell volume. Control and experimental groups, equal in number and of the same average body weight at the beginning of experiment, were studied at each age period. Half

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of each litter served as the controls in the youngest group studied. No litter exceeded 6 in number. Erythropoietin was injected daily for 10 days. Twenty-four hours after last injection, total red cell volumes were measured by the labeled red cell dilution technic(2). Hematocrits and hemoglobin concentrations were also determined. In the young animal total red cell volume is normally increasing due to growth. Total red cell volumes and total circulating hemoglobins of the animals receiving erythropoietin were thus compared with those of their respective controls, to determine whether there was any gain in total red cell volume in excess of that due to growth and only as a result of injection of erythropoietin. Since the anemia, which normally occurs in young rats, is most severe at 15-20 days after birth, 12-day-old rats were chosen for the youngest group so that the 10-day injection period would fall within the anemic period.

The effectiveness of erythropoietin was also measured by the red cell uptake of Fe^{59} (5) both in normal adult and in 14-day-old rats. Erythropoietin was administered for 2 days, and on the third day Fe^{59} was given intraperitoneally. Eighteen hours later a sample of blood from the aorta was counted. The results were expressed as per cent of administered Fe^{59} appearing in peripheral blood, using a value for the blood volume appropriate to the age of the animal. Measurements of blood volume gave values of 5.0 and 6.2% of body weight respectively for the adult and 14-day-old rat. Because of the possibility that young rats are producing red cells at a maximum rate, it was decided to test the effect of erythropoietin on such animals after depressing their red cell production by hypertransfusion. The same erythropoietin and Fe^{59} injection schedules were used except that prior to injection of erythropoietin the rats were injected intraperitoneally for 2 days with 1 ml of whole blood from an adult donor. Only animals with an hematocrit that exceeded that of normal adult rats (45%) were included. A value for blood volume of 7.2 ml per 100 g of body weight was used in the hypertransfused rat to express the results as per cent of administered Fe^{59}. This value had been measured previously in rats of the same age and hypertransfused in the same way.

The erythropoietin was obtained by ultrafiltration of urine through a collodion membrane from 2 anemic patients. The method of extraction has been described(6,7). The erythropoietin used in the red cell volume study was obtained by ultrafiltration of urine from an hypoplastic anemic patient, a summary of whose history has been published (6). The erythropoietin used in the Fe^{59} red cell uptake assay was obtained by ultrafiltration of urine from a patient with chronic bleeding from congenital telangiectases. A summary of the case history is to be published elsewhere. The erythropoietin was dissolved in saline and administered subcutaneously at a dose of 2 mg (0.2 ml) daily for 10 days for the blood volume assay, or 1 mg (0.2 ml) daily for 2 days for the Fe^{59} red cell incorporation assay. All controls received an equivalent volume of saline.

All rats were fed a complete laboratory diet.†

Results and discussion. The animals in all of the 5 groups responded to the erythropoietin with significant increases in total red cell volume and total circulating hemoglobin with the exception of the rats which were 12-days-of-age at beginning of experiment. Results of the red cell volume study are summarized in Table I. Since estimations of hemoglobin concentrations as well as hematocrit determinations were made, the value for total circulating hemoglobin as well as total red cell volume was calculated. In the 230- and 470-day-old rats, the percentage increase in hemoglobin is less than the percentage increase in red cell volume, indicating a degree of hypochromia in the cells produced under the influence of erythropoietin. This difference is even greater in the 23- and 87-day-old rats. Such a difference has been observed in the erythropoietic response

† The diet was obtained from Simonsen Laboratories, Gilroy, Calif. It consisted of 59.0% wheat, 11.7% skim milk, 11.2% casein, 11.2% rice bran, 3.3% vegetable oil, 1.3% $CaCO_3$, 0.7% NaCl, and vitamin and mineral mixtures to make up 100%.

TABLE I. Response of Male Rats of Various Ages to Treatment for 10 Days with 2.0 mg Daily of Human Urinary Erythropoietin.

Age, days	Treatment	No. rats	Wt, g (final)	Hct, %	Hgb, g/100 ml	Total red cell vol ml	% change	p*	Total circulating hemoglobin g	% change	p
12–22	2 mg "E"	12	49	28.0	7.1	.88	4.8	.5	.220	.7	.9
"	Control	12	52	25.4	6.7	.84			.219		
23–33	2 mg "E"	10	93	44.6	12.6	2.72	18.8	<.001	.769	11.1	<.02
"	Control	10	99	37.1	11.2	2.29			.692		
87–97	2 mg "E"	10	320	54.8	15.7	9.19	21.1	<.01	2.62	9.2	<.05
"	Control	10	336	46.7	14.8	7.59			2.40		
230–240	2 mg "E"	10	400	55.2	16.7	10.70	22.1	<.001	3.24	17.8	<.001
"	Control	10	407	47.4	14.9	8.76			2.75		
470–480	2 mg "E"	7	511	57.5	16.9	13.43	24.7	<.001	3.95	18.3	<.01
"	Control	7	519	48.6	15.1	10.77			3.34		

* Fisher's t test.

to hypoxia (4). Since the same total dose of erythropoietin was injected into the rats in the various age groups, the dose per unit body weight increased from the oldest to the youngest animals studied. Of the 4 groups which responded however, the absolute and relative increase is greatest in the oldest animals studied, *i.e.*, the ones that received the lowest dose per unit body weight. It would appear that the older the animal, the more responsive it is to erythropoietin.

It is interesting to note that the erythropoietic response to hypoxia is similar to that resulting from erythropoietin administration in at least 2 respects. Both stimuli fail to increase erythropoiesis in very young rats, and the response seen in older rats to either hypoxia or erythropoietin is characterized by production of slightly hypochromic cells.

Table II presents the results of Fe^{59} red cell uptake studies. One mg of erythropoietin was sufficient to elicit a significant increase in the red cell incorporation of Fe^{59} in normal adult male rats. When this dose of erythropoietin was injected into normal 14-day-old rats, no increase in incorporation of Fe^{59} into red cells was seen. When such young animals were hypertransfused, incorporation of Fe^{59} into red cells was reduced from the normal value of 37.7 to 24.6%. Injection of erythropoietin into such animals resulted in a significant increase, giving a value of 45.8%.

These data support the concept that erythropoiesis is very active in young rats during the period of neonatal anemia. Such animals cannot be stimulated to any greater extent with erythropoietin. Rats of this age do not respond erythropoietically to hypoxia. That young rats are capable of responding to erythropoietin, however, is shown by the fact that when erythropoiesis is depressed by hypertransfusion, they respond to injection of erythropoietin by an increased incorporation of radioiron into red cells.

Conclusions. Erythropoietin of human

TABLE II. Red Cell Incorporation of Radioiron in Normal and Hypertransfused Rats Following Injection of Erythropoietin.

	Age, days	No. rats	Hematocrit, %	Fe^{59} uptake in red cells, %	p*
Normal control	57	10		14.4	<.001
" 1 mg erythropoietin	57	10		31.0	
" control	14	7	22.0	37.7	
" 1 mg erythropoietin	14	6	24.5	38.6	
Hypertransfused control†	14	4	51.0	24.6	<.001
" 1 mg erythropoietin	14	5	52.7	45.8	

* Fisher's t test. † 1 ml whole blood I.P. for 2 days prior to inj. of erythropoietin.

urinary origin was injected into groups of rats of various ages, after which both measurements of total circulating red cell volume and Fe^{59} red cell incorporation were made. Total red cell volume of young rats throughout the period of neonatal anemia did not increase in response to administration of erythropoietin, whereas it did increase in all older groups. According to this assay, the rat most sensitive to erythropoietin was the oldest rat studied. Using the Fe^{59} red cell incorporation assay, no increase in erythropoiesis was observed in normal young rats (14 days of age) following erythropoietin administration. However, when erythropoiesis was depressed in young rats by hypertransfusion, red cell uptake of Fe^{59} was greatly reduced. When such hypertransfused rats were injected with erythropoietin, red cell incorporation of Fe^{59} was significantly increased.

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Organic Acid Excretion, Enhanced Calcium Absorption and Body Fat of Rats Fed Incompletely Digested Carbohydrates.* (26411)

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A relatively low content of body fat has been found in rats fed diets containing the incompletely digested carbohydrates, lactose, sorbitol, cellobiose or raw potato starch(1). The mechanisms involved are not known. Other characteristic effects associated with feeding of poorly digested carbohydrates include an initial transient diarrhea, enlargement of the cecum, establishment of an aciduric bacterial flora in the intestine, and enhanced gastrointestinal absorption of calcium and other alkaline earth elements(2). Recently Fournier and Digaud(3) have reported an increased urinary excretion of α-ketoglutaric, citric, aconitic, malic and succinic acids but not of pyruvic or hippuric acid. The present study investigated the possibility that the increased excretion of acids of the Krebs cycle is a consequence of metabolic alterations that result in a decreased deposition of body fat.

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Methods. Male weanling Sprague-Dawley rats, individually caged, were fed diets whose basal composition consisted of casein 24, carbohydrate 52, fat mixture 20, salts 4 and adequate amounts of vitamins(1). Twenty-four hour urine samples were collected under toluene. Body fat content was determined by whole carcass analysis(1) or estimated from the weight of the epididymal fat pads. The weight of the cleaned cecum was determined as an index of the digestibility of the dietary carbohydrate. Total organic acids in the urine were determined by the method of Van Slyke and Palmer(4). As this method is not specific, determination of keto acids by the simple and rapid method of Friedemann and Haugen(5) was routinely used to follow the effect of dietary carbohydrate on organic acid excretion. The organic acids were identified by silica gel chromatography. A 10-fold concentration of lyophilized urine in 2 N H_2SO_4 was mixed with an appropriate amount of dry silic acid and placed on top of a column prepared, and then eluted, by the

procedures of Dajani and Orten(6). Urinary Na, K and Ca were determined by standard flame photometric procedures; Ca was first separated as the oxalate. Mg was determined colorimetrically(7).

Results. Rats fed a diet containing lactose excreted 3 to 4 times more organic acids than those fed a glucose diet. Values of 0.89 and 0.84 meq/100 g/day were found for lactose-fed rats at the second and ninth week of feeding. At these same periods the glucose-fed animals excreted 0.26 and 0.22 meq/100 g/day. Excretion of organic acid, expressed as keto acid, was constant in terms of body weight during the first 9 weeks of experiment; keto acid excretion of the lactose group fell in the range of 6.8-10.3 mg/100 g/day, the glucose group 1.7-2.9 mg/100 g/day.

Fractionation of urine on silic acid columns revealed that the predominant organic acids were citric, α-ketoglutaric, succinic and fumaric acids, confirming the observations of Fournier and Digaud(3). A typical chromatographic profile of a urine sample from a lactose-fed rat is presented in Fig. 1. No qualitative difference was noted in the organic acid pattern of urine from lactose and glucose-fed rats although positive identification of all of the 9 or 10 peaks usually found was not attempted.

The relationships between the digestibility of the dietary carbohydrate, organic acid excretion and body fat content are presented in the experiment outlined in Table I. Rats fed the readily digested and absorbed carbohydrates, sucrose, glucose and galactose, excreted relatively small amounts of organic

TABLE I. Dietary Carbohydrate, Body Fat and Organic Acid Excretion.

Carbohydrate	Cecum (g/100)	Keto acid† (mg/day)	Body fat (%)
Glucose	.22	3.1 ± .2‡	14.2 ± .6‡
Sucrose	.24	3.5 ± .1	13.0 ± .4
26% galactose*	.23	2.7 ± .2	13.3 ± 1.7
Lactose	.74	10.9 ± .9	9.8 ± .6
15% cellobiose*	.58	5.1 ± .7	9.8 ± .6
26% raw potato starch*	.65	8.9 ± .3	10.8 ± .5

6 wk experiment, 7 rats/group.
* Glucose added to 52% total carbohydrate.
† Avg of 4 daily averages during 2nd to 6th wk.
‡ ± S.E.

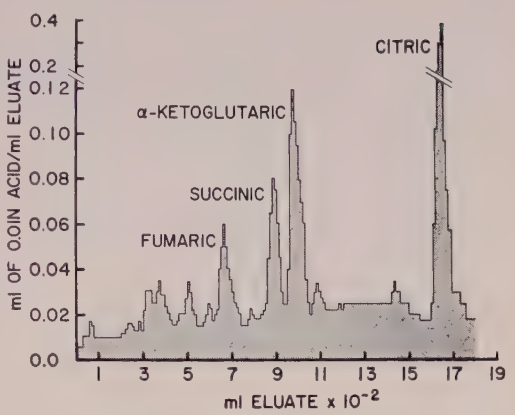

FIG. 1. Silica gel chromatogram of urinary organic acids of lactose-fed rat. 2.2 ml of 10-fold concentrated urine on column 2 × 28 cm, gradient elution, $CHCl_3$ to 45% ter-amyl alcohol in $CHCl_3$, 1 liter each solvent.

acids and had more body fat than rats fed the incompletely digested carbohydrates, lactose, cellobiose and raw potato starch. In every case the differences in body fat content and acid excretion between the readily digested and the incompletely digested carbohydrates were of statistical significance, $p = 0.05$ or less (Student's t test).

The increase in organic acids in urine of the rats fed incompletely digested carbohydrate did not appear to originate from the increased numbers of bacteria in the intestinal tract, since feeding of antibiotics did not lower keto acid excretion. Addition of 50 mg of chlortetracycline or a combination of 200 mg each of bacitracin and polymyxin B to a kilogram of the lactose diet resulted in an average excretion of 20.4 mg/day and 17.1 mg/day of keto acids respectively as compared to 20.9 mg/day excreted by a control group. It had also been observed(8) that supplementation of a lactose diet with chlortetracycline did not influence body fat content.

The increase in urinary organic acid during a metabolic acidosis is characterized by a concomitant increase in ammonia. Rats fed lactose-containing diets excreted less ammonia than glucose controls; 3.9 *vs.* 5.9 mg ammonia N/100 g/day in a typical experiment. The urinary pattern was more suggestive of a compensated alkalosis and since it is well known that the feeding of lactose

TABLE II. Urinary Excretion of Anions and Cations.

Constituent	Lactose (meq/day)	Glucose (meq/day)
Na	.35	.30
K	.99	.74
Ca	.52	.03
Mg	.08	.04
Cl	1.02	.71
HPO_4	.22	.46
SO_4	.13	.08
Organic acid	2.21	.48
Keto acid	.21	.03

Avg of 3 daily averages of 5 rats/group after 5 wk on diet.

will stimulate gastrointestinal absorption of calcium and other alkaline earth elements (2) it seemed likely that the increased organic acid excretion was the result of a renal mechanism for neutralization of the additional calcium excreted. The increased excretion of organic acids, particularly citric acid, resulting from administration of alkalinizing salts, has been extensively studied in the rat(9-13).

An analysis of the principal ionic constituents of urine samples from lactose and glucose-fed rats (Table II) yielded data in accord with this concept. Urine from the lactose-fed rats contained about 15 times more calcium, with little difference in the content of the other cations. Of the anions, only the organic acid fraction was correspondingly increased.

The results of feeding tests (Table III) offered further evidence that the increase in urinary organic acids was involved in acid-base balance. In Exp. 1, excretion of organic acids was shown to be directly related to salt content of the diet. Increasing or decreasing the salt content, irrespective of the nature of carbohydrate, resulted in a corresponding change in organic acid excretion. In Exp. 2 it was demonstrated that the effective ingredient of the salt mixture was $CaCO_3$; 54% of the Hubbell, Mendel, Wakeman salt mixture(14) is $CaCO_3$. Addition to the diet of $CaCl_2$, a calcium salt of a fixed anion in contrast to the metabolizable anion, carbonate, resulted in an organic acid excretion less than that of the control, Exp. 2, Table III. Analysis of the urine of the rats fed additional $CaCO_3$ or $CaCl_2$, Table IV, showed an equal

TABLE IV. Effect of Dietary Calcium Salts on Urine Constituents.*

Salt†	Ca (meq/day)	Cl (meq/day)	Keto acid (meq/day)
2.2% $CaCO_3$	1.0	.6	.28
2.2% $CaCl_2$	1.1	2.8	.04
None	—	—	.16

6 rats/group, 6 wk experiment.

* Avg of 3 daily averages during 5th to 6th wk.

† Added to diet containing lactose and 2% salt mixture.

excretion of calcium but in the rats fed $CaCl_2$, with an excess of anion, chloride, to be eliminated, organic acid excretion was depressed. The effect of feeding other alkalinizing salts, *i.e.*, salts of a fixed base and metabolizable anion, was studied in Exp. 3, Table III. Sodium citrate and sodium acetate greatly stimulated acid excretion; an equivalent amount of acetate as triacetin had no

TABLE III. Effect of Dietary Salts on Organic Acid Excretion.

Diet characteristic	Keto acid (mg/day)* Exp. 1	Exp. 2	Exp. 3
Glucose, 4% salt mixture	4.2	—	
" 6% " "	6.7	—	
Lactose, 2% " "	11.6	16.3	
" 4% " "	23.0	22.6	
" 6% " "	32.9	27.5	
" 2% " " + 2.2% $CaCO_3$	—	20.0	
" 2% " " + 2.2% $CaCl_2$	—	2.7	
Glucose, 4% " " + 3.6% Na acetate	—	—	45.1
Lactose, 4% " " + 3.2% triacetin	—	—	1.9
" 4% " " + 4.3% Na citrate	—	—	40.2
" 4% " " + 2.6% NaCl	—	—	3.7

* Avg of 4 daily averages during 2nd to 6th wk. 7 rats/group, 6 wk experiment.

TABLE V. Nonrelationship of Body Fat and Calcium and Acid Excretion.

	Diet characteristic	Epididymal fat body (g/100 g)	Carcass fat (%)	Keto acid* (meq/day)	Ca* (meq/day)
Exp. 1	Glucose, 4% salt mixture	2.0	16.6	.04	—
	Idem + 3.6% Na acetate	1.8	16.7	.61	—
	Lactose, 4% salt mixture	1.2	11.6	.21	—
Exp. 2	Glucose, 4% salt mixture	2.0	—	.08	.06
	Idem + 6% salt mixture	2.0	—	.28	.15
	" + 3.5% Ca and Mg salts†	2.0	—	.25	.31

* Avg of 4 daily averages during 3rd to 7th wk.
† Amounts in 6% total salt mixture. 6 rats/group, 7 wk experiment.

effect. The neutral salt, NaCl, yielded the expected low value.

The continuous excretion of abnormally large amounts of organic acids for 6 or 7 weeks was not in itself responsible for the low deposition of carcass fat. In Exp. 1 of Table V, rats fed a glucose diet with added sodium acetate had a high body fat content despite daily excretion of 45 mg of keto acid, about twice that of lactose-fed rats. Neither was the low body fat content of the lactose-fed rat caused by the increased absorption, transport and excretion of calcium. In Exp. 2 of Table V, urinary keto acid and calcium excretion of rats fed a glucose diet was enhanced by increasing the amount of the total salt mixture from 4 to 10% of the diet, or by adding calcium and magnesium salts in amounts supplied by the additional 6% salt mixture. No difference was found in the deposition of body fat as measured by the weights of the epididymal fat pads.

Discussion. The increased urinary excretion of acids of the Krebs cycle, observed in rats fed diets containing incompletely digested carbohydrates, is not a manifestation of an altered metabolism that results in a lowered deposition of body fat but appears to be a renal mechanism for neutralization of increased urinary calcium, an increase resulting from enhancement of gastrointestinal absorption of calcium by these carbohydrates (15,16). The experiments of the present study have shown that organic acid excretion is influenced by the amount of salt mixture in the diet and more specifically, by the nature of the calcium salts in the salt mixture. When calcium is ingested as calcium carbonate, the carbonate is metabolized leaving an excess of base to be neutralized by organic acid. With $CaCl_2$ in the diet, since a major fraction of the calcium is excreted in the feces, chloride is in excess and renal elaboration of organic acid is suppressed. It is known that organic acid excretion plays a more dominant role in acid-base balance in the rat than in man (12); little bicarbonate is found in rat urine. The renal origin of these acids has been indicated by the findings of several investigators that, with the injection of alkalinizing salts, an increase of citrate is found in renal tissue and urine but not in blood(10,11) and recent evidence(13) further suggests that the excretion rate of citrate, and probably all the acids of the tricarboxylic acid cycle, is dependent not on renal-tissue levels but on renal acid-base balance.

The results of the experiment presented here offer no enlightenment on the nature of the relationship between carbohydrate digestibility and body fat content. Attempts to reduce the body fat content of glucose-fed rats by inducing a urinary excretion of organic acid by feeding sodium acetate, and of calcium by increasing the salt content of the diet, had no effect on fat deposition.

Summary. 1. Rats fed diets containing the incompletely digested carbohydrates, lactose, cellobiose and raw potato starch, excreted larger amounts of organic acids and had a lower content of body fat than rats fed glucose, sucrose or galactose. 2. The predominant urinary organic acids were identified by silica gel chromatography as citric, α-ketoglutaric, fumaric and succinic acids. 3. Organic acid excretion could be increased by increasing the amount of dietary salt mixture or by adding $CaCO_3$ but not $CaCl_2$ to the

diet. 4. This latter finding, in conjunction with the results of urinary analysis of ionic constituents, indicated that the increased excretion of organic acid is not a consequence of an altered metabolism that decreases body fat deposition but is a renal acid-base mechanism for neutralization of increased urinary calcium, an increase resulting from the enhancement of gastrointestinal absorption of calcium by incompletely digested carbohydrates.

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Leucinamidase Activity Associated with Influenza-Parasite Complexes in Pigs.* (26412)

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Activity of amino acid amidases is increased in homogenates prepared from bovine kidney monolayered tissue cultures when infected with the virus of infectious bovine rhinotracheitis virus. Leucinamidase was the most active of the 5 enzymes studied(1). Amide-N^{15} from either leucinamide or glutamine was incorporated into cellular nucleic acid in both normal and virus infected cells but activity was greater in the infected cells (2). Studies have been extended to evaluation of serum leucinamidase activity in pigs. The enzymic activity in serum of pigs which were infected with swine influenza virus. *Ascaris suum,* and lungworms (*Metastrongylus pudendotectus* and *M. apri*) which harbor influenza virus is herein reported.

Materials and methods. One week-old pathogen-free, colostrum-deprived pigs housed in modified Horsfall-Bauer isolation units as previously described were used as the experimental host(3). Eight littermate pigs were utilized in pairs for each of 4 experimental conditions. These were: 1) Uninoculated controls; 2) Inoculated with Shope's strain 15 of swine influenza virus; 3) Fed infective eggs of *Ascaris suum;* and 4) Fed infective larvae of swine lungworms originating from influenza-infected pigs. Fifteen days after the lungworm larvae were given to the pigs *Ascaris* eggs were fed to provoke the masked influenza virus(4). Methods used for preparation and inoculation of virus and parasites(5,6,7) and for evaluation of

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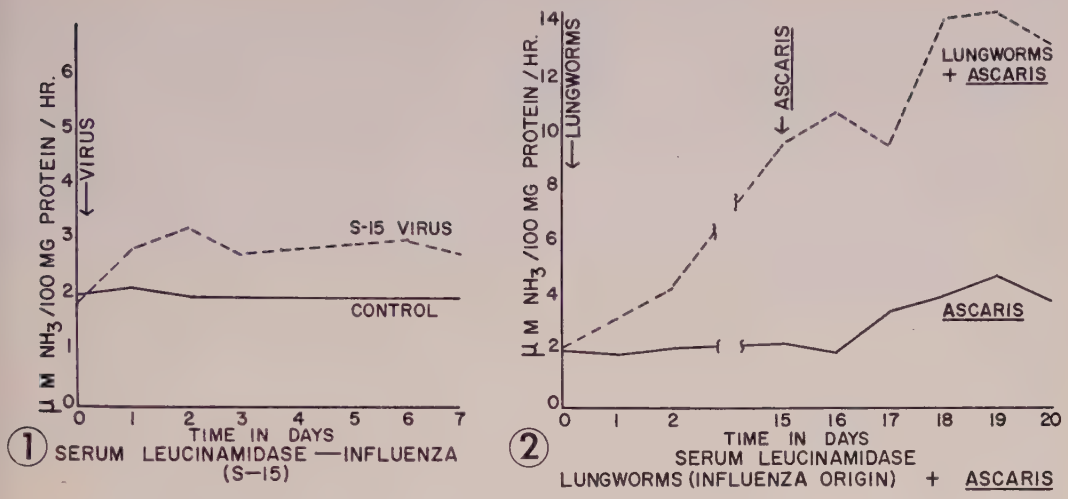

FIG. 1. Leucinamidase activity in serum of normal pigs and pigs infected with swine influenza (S-15) virus.

FIG. 2. Leucinamidase activity in serum of pigs infected with *Ascaris suum* larvae and pigs infected primarily with lungworm larvae of influenza origin and secondarily with *A. suum* larvae.

leucinamidase(8) have been described. Serum was obtained from blood drawn from the anterior vena cava.

Results. Leucinamidase activity remained relatively constant in the uninfected control pigs at approximately 2 μ M of NH_3/hour/100 mg serum protein (Fig. 1). Activity was slightly increased in the influenza infected pigs with values near 3 μ M. Body temperatures remained normal in the uninoculated pigs but were slightly increased in virus infected pigs. At necropsy 7 days after infection, lungs of control pigs were normal whereas the lungs from influenza virus infected pigs showed typical pneumonic areas.

The pigs which received embryonated *Ascaris* eggs on the 15th day showed amidase activity similar to the uninoculated controls up until 2 days after feeding *Ascaris* eggs (Fig. 2). A noticeable rise in activity began at the time larval migration was at its height. Temperatures remained normal. Lungs at necropsy showed only hemorrhages typical of *Ascaris* larval migration.

Pigs inoculated with lungworms of influenza origin showed the greatest amount of serum leucinamidase activity(Fig. 2). Typical swine influenza lesions and lungworms were present in the lungs at necropsy. Swine influenza virus was isolated from the lungs by egg inoculation and specifically identified by serum neutralization test. Increased leucinamidase activity in these pigs appears to be accumulative from the concerted effect of an established lungworm infection, migrating *Ascaris* larvae, and an active swine influenza virus infection.

Discussion. Increased leucinamidase activity in swine appears to be associated with cellular damage whether caused by virus or parasites. Other observations in this laboratory (unpublished) show little or no association of increased activity to febrile response. Virus pneumonia of pigs (VPP), which is a chronic respiratory disease, induces no temperature reaction yet elicits increased leucinamidase activity. Contrarily, swine edema disease which causes severe endothelial and myocardial damage, induces temperatures 107°-108°F but leucinamidase activity increases rapidly to 6-8 μM before temperature increases are evident. Hog cholera follows a similar pattern.

An association of leucinamidase to host resistance is implied in these experiments. Lungworms of influenza origin are incapable of producing influenza in pigs without some external stimulus. Migrating *Ascaris suum* larvae with associated increased leucinamidase activity served as a provoking stimulus

to free lungworm-bound influenza virus. Aqueous extracts of *Ascaris* also stimulate increased leucinamidase to provoke the virus (5). This eliminates tissue damage by migrating larvae as necessary to provoke influenza infection.

No tests have been made on serums from pigs infected experimentally with pathogenic bacteria or leptospira. These obviously must be made.

Enzyme activity is a sensitive health indicator. In our laboratory, 2-3-day-old pigs, infected with *Ascaris* larvae, show a rise followed by a partial decline in leucinamidase activity when the larvae pass through the intestine, when they pass through the liver (most marked), and again when they pass through the lungs. Established base lines of serum enzyme activity might be useful indicators of health in other species. Amino acid amidase activity might indicate latent infections or parasitism which would eliminate potential hosts from experiments that would be voided by a complex etiology.

Summary. A moderate increase in serum leucinamidase activity occurred in colostrum-deprived pathogen-free pigs experimentally infected with swine influenza virus. Activity also increased during migration of *Ascaris suum* larvae through the liver and again when the larvae reached the lungs. Greatest leucinamidase activity was stimulated when influenza virus was provoked from its latent lungworm state by migrating *A. suum* larvae.

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Serum and Urine 17-Hydroxycorticosteroid Levels Following Small Oral Doses of Synthetic Analogs of Hydrocortisone.* (26413)

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Earlier reports from these laboratories enumerated changes in body fluid 17-hydroxycorticosteroid (17-OHCS) levels following oral administration of synthetic analogs of hydrocortisone(1,2). Parotid fluid steroid findings have reliably reflected serum and urine results. However, the *magnitude* of steroid response differed with each drug, even though all doses were 50 mg. The present study was designed to investigate this variability in steroid response by employing smaller doses of hydrocortisone analogs.

Materials and methods. Volunteer subjects were 281 young adult male airmen, all judged physically qualified for military service by recent medical examination. Diet, environmental exposure, times of arising and retiring, and other conditions were virtually identical in all instances. Each day, 16 subjects were randomly divided into experimental and control groups, and these groups further divided into 4-hour and 7-hour groups. At 0800, blood was collected by venipuncture and the serum retained. A force-voided urine specimen was discarded. A second blood sample was collected at 1200 or 1500 hours, *i.e.*, either 4 or 7 hours after the initial sampling. Urine was collected over the respective 4 or 7-hour periods. The drugs

* The opinions expressed herein are those of the authors and are not to be construed as representing USAF policy.

TABLE I. Free 17-Hydroxycorticosteroid Levels in Serum Following Oral Administration of Synthetic Analogs of Hydrocortisone.

Drug	0800-1200 hr					0800-1500 hr				
	Free 17-OHCS, γ %					Free 17-OHCS, γ %				
	No.	Time	Mean	S.D.	P	No.	Time	Mean	S.D.	P
Control	57	0800	13.78	4.86	—	58	0800	12.02	4.37	—
		1200	12.50	5.63			1500	10.44	4.63	
Prednisolone, 10 mg	12	0800	11.32	2.82	<.01	16	0800	14.31	2.88	N.S.
		1200	18.62	4.12			1500	12.16	3.64	
Decadron, 2.5 mg	20	0800	11.83	4.08	<.01	18	0800	12.37	3.97	<.01
		1200	6.67	3.02			1500	4.07	2.24	
Triamcinolone, 10 mg	12	0800	11.44	4.00	<.01	16	0800	15.01	6.04	<.01
		1200	3.72	2.64			1500	3.01	2.19	
Medrol, 10 mg	19	0800	12.17	3.08	N.S.	19	0800	13.84	4.23	<.01
		1200	10.02	4.40			1500	6.15	2.70	
2-CH_3-F-F, 1.5 mg	19	0800	13.98	4.61	<.01	15	0800	12.24	3.80	<.01
		1200	6.39	2.71			1500	3.31	2.04	

under study were administered orally immediately after the initial blood sampling. The compounds† and relatively comparable dosages employed were as follows: prednisolone (delta-1 hydrocortisone) 10 mg; medrol (delta-1, 6-methyl hydrocortisone) 10 mg; triamcinolone (delta-1, 9-alpha-fluoro, 16-hydroxy hydrocortisone) 10 mg; decadron (delta-1, 9-alpha-fluoro, 16-methyl hydrocortisone) 2.5 mg; and 2-methyl, 9-alpha-fluorohydrocortisone (2-CH_3-F-F) 1.5 mg.

Concentration of free 17-OHCS in serum was determined by the method of Peterson *et al.*(3), utilizing a methylene chloride extraction procedure. Total 17-OHCS levels in urine were determined by a modification combining the butanol extraction methods of Reddy(4) and Clayson(5). Both blood and urine technics are based upon the Porter-Silber(6) color reaction between 17, 21-dihydroxy-20-ketosteroids and phenylhydrazine in sulfuric acid.

Results and discussion. The effect of the drugs on the free 17-OHCS concentration in serum is shown in Table I. Serum steroid values at 0800 hours were relatively constant throughout the experiment, as were control values at 1200 and 1500 hours. Decadron, triamcinolone, and 2-CH_3-F-F dosage prompted highly significant decreases at 1200 and 1500 hours in the concentration of the free steroids in serum. Prednisolone produced a significant steroid increase at 1200 and a return to normal level at 1500 hours. Administration of medrol had little effect at 1200; however, a significant decrease in steroid concentration was observed at 1500. There was considerable variation between drug groups in magnitude of response, ranging from a decrease of 12.00 γ % at 1500 for triamcinolone to an increase of 7.30 γ % at 1200 for prednisolone.

Data on urinary excretion of total 17-OHCS are presented in Table II. Prednisolone and medrol administration prompted marked increases in urine steroid levels during the 4-hour and 7-hour collection periods. Following decadron and triamcinolone dosage 17-OHCS values were not significantly different from control values. A highly significant decrease in urinary steroid excretion rate was observed following dosage with 2-CH_3-F-F.

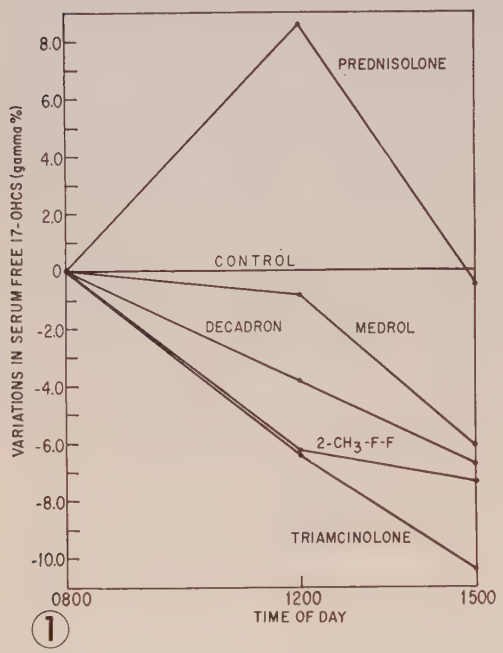
Relative differences in 17-OHCS levels in serum and urine are seen in Fig. 1 and 2, respectively. Changes in the 2 fluids tended to be parallel for each except medrol. A significant decrease in serum steroid concentration followed medrol dosage, while a marked increase in urinary 17-OHCS was found.

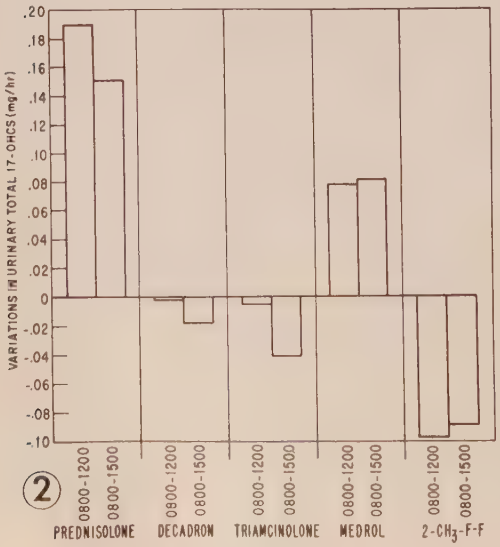
The divergent responses produced by the drugs under study suggest that the absorption and/or metabolic degradation of the

† Drugs furnished by Lederle Laboratories, Merck, Sharp and Dohme Co., and Upjohn Co.

TABLE II. Total 17-Hydroxycorticosteroid Levels in Urine Following Oral Administration of Synthetic Analogs of Hydrocortisone.

	0800-1200 hr Total 17-OHCS, mg/hr				0800-1500 hr Total 17-OHCS, mg/hr			
	No.	Mean	S.D.	P	No.	Mean	S.D.	P
Control	57	.40	.17	—	58	.33	.11	—
Prednisolone, 10 mg	12	.59	.19	<.01	16	.48	.14	<.01
Decadron, 2.5 mg	20	.40	.16	N.S.	18	.32	.14	N.S.
Triamcinolone, 10 mg	12	.40	.15	N.S.	16	.29	.10	N.S.
Medrol, 10 mg	19	.48	.10	<.05	19	.41	.14	<.05
2-CH_3-F-F, 1.5 mg	19	.30	.13	<.05	15	.24	.09	<.01





various compounds proceed at different rates or by entirely different pathways. Prednisolone prompted markedly increased steroid levels in both serum and urine, while the methylated derivatives (medrol, decadron, and 2-methyl, 9-alpha-fluoro-hydrocortisone) led to obvious adrenocortical suppression with significantly lower 17-OHCS concentrations in serum. The last two drugs also produced lower steroid levels in urine.

Ely and co-workers(7) studied the effect of orally administered prednisolone on plasma free 17-OHCS, and concluded that the metabolism of prednisolone proceeded at a rate slower than that of hydrocortisone. In "normal", hospitalized patients, Kupperman *et al.*(8) found an increased 17-OHCS excretion in urine following prednisone therapy. They concluded that this increase actually due to the presence of the administered steroid, more than offset the associated endogenous 17-OHCS suppression. These workers also found a decreased steroid excretion in patients dosed with medrol and triamcinolone caused, they felt, by the drugs not being metabolized into 17-hydroxysteroids.

In contrast to certain of the urinary findings of Kupperman *et al.*(8), who gave a 32 mg daily drug dose for a 5-day period, medrol was found in our studies to promote an increased steroid excretion while causing a decrease in serum levels. Since the urinary steroid procedure employed measures *total* 17-OHCS, as opposed to the *free* 17-OHCS

FIG. 1. Relative changes in serum free 17-OHCS levels following oral administration of synthetic analogs of hydrocortisone.

FIG. 2. Relative changes in urinary total 17-OHCS levels following oral administration of synthetic analogs of hydrocortisone.

assay in serum, a variance in rate of steroid conjugation may be implicated in the findings with this 6-methyl derivative of prednisolone. A study of free 17-OHCS levels in urine under comparable experimental conditions is being made to pursue this possibility further.

As reported earlier(1,2,9,10), triamcinolone does not give a Porter-Silber reaction. Thus the drop in measurable 17-OHCS signifies suppression of endogenous adrenocortical secretion.

Summary. The influence of small oral doses of hydrocortisone analogs on serum and urine 17-hydroxycorticosteroids was studied in 281 human subjects. With the exception of medrol, the drugs produced changes in 17-OHCS which tended to be parallel in serum and urine. The drugs differed greatly in response produced.

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Influence of Body Weight and Growth Rate on Nitrogen and Electrolyte Excretion in Rats.* (26414)

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Fasting weight loss in growing rats decreases with age and, therefore, with size(1). Weight loss is probably also a function of the higher growth rates of the younger rats. The resultant of these forces (growth rate and attained body weight) should be reflected in excretion rates of nitrogenous compounds and electrolytes. This possibility was examined with young male rats of different body weights and growth rates as subjects. The influence of differences in food intake was minimized by making the comparison on each day of a 3-day fast.

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Experimental. Twelve Sherman strain male rats between 67 and 70 days old (220 g) and 12 between 110 and 113 days old (265 g) constituted the light and heavy groups (L and H), respectively. All were raised in an animal room at 24°C. In the 2-week period before the experiment, Group L rats were gaining at the average rate of 3.0 g/day with food intake averaging 21.2 g/day (69.7 g/kg^{¾} body weight), while Group H rats were gaining at a much slower rate of .36 g/day with a food intake of 19.0 g/day (60.9 g/kg^{¾} body weight). The rats were placed into individual plastic-coated metabolism cages for 6 days (24°C). For the first 3 days food (Purina Dog Chow) and water were furnished *ad libitum* to accustom the animals to the cages. During the last 3

days they received only water. Urine samples (24-hour) were collected into acid on the 3 fasting days. These were pooled at random in each group into 4 samples from the 3 rats each. Clean cages were substituted daily. These samples were analyzed for 9 constituents by methods previously cited(2).

Results and Discussion. Significant overall group differences (including group x day interactions) occurred with most variables (Tables 1, 2, 3). As others have observed

TABLE I. Influence of Body Weight on Physiological Changes of Rats during Fasting. General variables.*

Variable	Wt group	Days (means) fasted 1	2	3	Error terms $\sqrt{MSe_1}$	$\sqrt{MSe_2}$	Pertinent significant "F" ratios bg	bd	g × d
Body wt (g)	L	198	191	171	14.7	3.997	55.	82.6	N.S.
	H	244	231	220					
Wt loss (g/24 hr)	L	24	7	20	3.56	8.12	N.S.	4.58	"
	H	21	13	11					
Wt loss (% of initial wt)	L	10.6	3.7	10.3	1.435	3.788	29.	N.S.	"
	H	8.0	5.3	4.7					
Water intake (ml/24 hr)	L	21	12	7	14.10	6.488	N.S.	9.84	"
	H	23	18	8					
Urine vol (ml/24 hr)	L	14	5	.5	9.772	6.738	"	7.57	"
	H	14	7	.8					
Ratio: Urine/Water	L	.69	.47	.06	.210	.214	"	12.	"
	H	.50	.32	.09					

* Means of 2 wt groups (light, L, and heavy, H) for 3 consecutive days of fasting are presented. Only significant "F" ratios are presented (N.S. = not significant; N.A. = not applicable, as where there was a significant interaction). $\sqrt{MSe_1}$ = mean square between subjects in the same group used to test between group differences; $\sqrt{MSe_2}$ = mean square pooled subjects × days interaction used to test the between day differences and the group × day interaction. Individual group means may be compared on the same day by Cochran's approximate t-test(3, p. 272).

TABLE II. Influence of Body Weight on 24-Hour Electrolyte Excretion by Rats during Fasting (meq).*

Variable	Wt group	Days (means) fasted 1	2	3	Error terms $\sqrt{MSe_1}$	$\sqrt{MSe_2}$	Pertinent significant "F" ratios bg	bd	g × d
Sodium	L	1.02	.41	.24	.0539	.0601	N.A.	N.A.	27.
	H	.65†	.38	.29					
Potassium	L	1.31	.70	.78	.1276	.0802	N.S.	127.	N.S.
	H	1.25	.68	.79					
Ratio: Na/K	L	.77	.59	.31	.0596	.07006	N.A.	N.A.	10.2
	H	.52†	.55	.37					
Magnesium	L	.21	.34	.24	.0386	.0293	"	"	7.44
	H	.14†	.30†	.23					
Calcium	L	.091	.070	.083	.00775	.00424	"	"	8.89
	H	.086	.082†	.086					
Ratio: Mg/Ca	L	2.3	5.0	2.9	.4808	.4130	"	"	4.92
	H	1.7†	3.6†	2.7					
Phosphate (as P)	L	1.18	1.20	1.16	.0566	.0245	14.	N.S.	N.S.
	H	.94†	.90†	.88†					
Ratio: Ca/P	L	.078	.060	.073	.0172	.00866	12.	"	"
	H	.092†	.093†	.098†					

* See footnote of Table I.
† Significant group difference (Cochran's approximate t-test(3, p. 272).

TABLE III. Influence of Body Weight on 24-Hour Excretion of Nitrogenous Compounds by Rats during Fasting (mg).*

Variable	Wt group	Days (means) fasted 1	2	3	Error terms $\sqrt{MSe_1}$	$\sqrt{MSe_2}$	Pertinent significant "F" ratios bg	bd	g × d
Urea	L	517	408	317	47.2	24.7	N.A.	N.A.	5.78
	H	546	353†	310					
Uric acid	L	2.4	2.6	2.3	.369	.158	N.S.	14.	N.S.
	H	2.1	2.4	2.0					
Creatinine	L	6.4	6.6	6.5	.541	.275	N.A.	N.A.	11.
	H	6.8	8.3†	7.7†					
Ratio: Uric acid/ Creatinine	L	.38	.36	.31	.0452	.0226	21.	18.	N.S.
	H	.31†	.28†	.26†					
Histidine	L	3.4	2.4	1.6	1.72	1.46	N.S.	37.	"
	H	2.8	2.0	1.7					

* † See footnotes of Table II.

(1), the lighter (and younger) rats lost more weight during the fast than the heavier ones, and they excreted slightly less total calcium (4) over the 3 days. Since they excreted more phosphate, their Ca/P ratios also were lower. The lighter rats excreted more magnesium, and the slight retention of calcium (mentioned above) becomes quite apparent in the elevated Mg/Ca ratios. The heavier rats retained more sodium on day 1 of the fast; this was also reflected in the Na/K ratio.

Since the younger rats were eating more, higher total loss of urea was to be expected. Likewise, their smaller muscle mass as well as their higher growth rate could explain their lower creatinine excretion. Also, since mean uric acid excretion was slightly elevated each day in these lighter rats, their uric acid/creatinine ratios were uniformly higher.

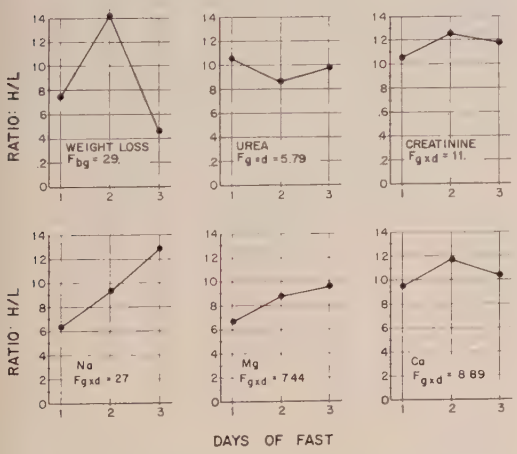

FIG. 1. Differential changes in fasting excretion rates of young male rats as a function of body wt and growth rate. Each value expressed as ratio of heavier to lighter rats; pertinent F-ratios (analysis of variance) are presented: F_{bg} = between the 2 groups, and $F_{g\times d}$ = interaction.

These differential day-to-day changes are illustrated in Fig. 1, where various ratios of heavier to lighter rats are presented. On succeeding fast days ratios for sodium and magnesium increased in near-linear manner from their initial low values. Calcium also was low initially, but increased temporarily on Day-2 to return nearly to unity by Day-3. Daily weight loss (percentage) followed the same pattern as calcium except that in this case on Day-3 it became very low. The creatinine ratio started high and increased further on Day-2. Although it decreased slightly on Day-3, it remained higher than the initial value. Urea on the other hand with the same initial ratio as creatinine fell on Day-2 and returned to unity on Day-3.

It may appear that excretion rates should be equalized by expressing the results on a body weight basis. Expressed on this basis, the group differences become quite large with variables such as magnesium, where the lighter group excreted more than the heavier rats, but they are minimized with variables such as phosphate where the converse is the case. Correlations of body weight with urinary variables are essentially unknown. In an earlier study with adult rats(5) correlations with body weight on the whole were quite low and many were not even significant (e.g., sodium,

calcium, phosphate, uric acid and creatinine). Furthermore, body weight decreases on succeeding days of the fast and possibly the intercorrelations also change. It does not seem justifiable, therefore, to express excretion rates of fasting rats on a body weight basis until much more information is available.

Conclusion. Young male rats that differed in body weight by only about 20% had significant ($p \leq .05$) metabolic differences during a 3-day fast. These included calcium, phosphate, urea, creatinine, the Na/K, Mg/Ca, Ca/PO_4 and uric acid/creatinine ratios. There was an almost 10-fold difference in their respective growth rates, however, which undoubtedly contributed to the differences. These group differences tended to disappear in almost all variables as the fast progressed. The various ratios proved to be quite sensitive to the group differences.

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A Hemolytic Phenomenon in Ewes Requiring *Leptospira pomona* Antigen and Antibody.* (26415)

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The properties of *Leptospira pomona* responsible for the hemoglobinemia and hemoglobinuria observed in field and experimental infections of cattle and sheep have not been defined(1,2,3,). *In vitro* studies have revealed the presence of hemolytic factors in the culture supernatant fluid(4,5,6). Little hemolytic activity was observed in preparations of washed disrupted cells(4,6). The hemolytic factor was thermolabile (inactivated at 56°C in 10 minutes) inhibited by specific antiserum, independent of complement and most active against erythrocytes of ruminants. Intravenous administration of the material produced marked and fatal hemolysis and hemoglobinuria in nonimmune lambs(7,8) Specific *L. pomona* antibodies inhibited hemolysis and protected lambs(7). The similarity of the effect of the hemolytic factor in lambs to the syndrome observed following active *L. pomona* infections has been reported(8).

During experimental infections in cattle and sheep, *L. pomona* has been isolated from the blood stream after the appearance of detectable specific antibodies. Ferguson *et. al.* (9) suggested that a hemolytic toxin was released from the leptospirae by the effect of a lytic antibody. Morse *et al.*(3) suggested a similar phenomenon as the cause of hemolytic anemia in experimental *L. pomona* infections of sheep.

Hemolysis and hemoglobinuria in cattle and sheep have occurred after the appearance of specific antibodies. Inhibition by specific leptospiral antibodies of the hemolysin from culture supernatant fluids suggests that another factor is responsible for hemolysis in actual infections. This preliminary investigation was undertaken to delineate the relation

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between *L. pomona* cell suspensions and hemolysis in immune sheep.

Materials and methods. Preparation of cell suspensions. *L. pomona* (strain LW) (10) was cultivated in Stuarts (Difco) medium containing 10% gamma-globulin-free sterile rabbit serum. Cultures were incubated for 10 to 12 days at 28°C, refrigerated at 5°C for 5 days and centrifuged at approximately 3500 g. The sedimented leptospirae were washed 3 times with approximately 15 cc sterile 0.85% saline, resuspended to approximately 1% of the original culture volume in sterile saline and stored at –30°C which rendered the leptospirae non-viable. The cells were disrupted by grinding in a motor driven, 40 ml Ten Broeck tissue grinder placed in an ice bath. After grinding for 15 minutes, no intact cells were observed in the resulting suspension by darkfield examination (525x).

Experimental animals were five apparently normal, mature, crossbred ewes. No *L. pomona antibodies* were detected in the sera of the ewes by modified agglutination-lysis test procedures(11). A single ewe, used in Trial 1, received an inoculum of 2.5 ml of culture of *L. pomona* (strain 3050) subcutaneously. Strain 3050 was originally isolated from sheep. Rectal temperatures were recorded daily through postexposure day 7. Hemocultures were prepared on postexposure days 5 to 7 inclusive, incubated at 28°C and examined by darkfield illumination (525x) at weekly intervals for one month. Serum titer was checked by the agglutination-lysis test. Seventy and 73 days post exposure the ewe was given 4.5 ml of disrupted cell suspension. In Trial 2, 3 ewes were inoculated with strain 3050 and one ewe received 2.5 ml of sterile isotonic saline. Postexposure procedures were the same as in Trial 1 until day 15. All 4 ewes were given 4.5 ml of disrupted cell preparation on postexposure day 15. On day 19, ewe 41 received 1½ ml and the other 3 ewes 3 ml intravenously. Hemoglobin values were determined as cyanmethemoglobin by optical density with a Bausch and Lomb Spectronic 20 colorimeter at 540 mμ. Hematocrit values were ascertained in Wintrobe hematocrit tubes.

Results. The exposure to *L pomona* (strain 3050) in Trial 1 produced a mild leptospirosis demonstrated by recovery of the organism in hemocultures and agglutination-lysis reactions in serum dilutions of 10^{-3}. Hemoglobinuria was observed 48 hours after subsequent administration of the cell suspension, and persisted until the ewe died approximately 60 hours later. Ten hours before death the hemoglobin value was 2.7 g per 100 ml of blood and packed cell volume 7.0%. The terminal agglutination-lysis reaction was 50% complete in a serum dilution of 10^{-4}.

In Trial 2, leptospirae were isolated in hemocultures from 2 of 3 exposed ewes. Agglutination-lysis reactions were observed in serum dilutions of 10^{-2} on postexposure day 9. Maximal rectal temperatures observed from postexposure days 5 to 9 were 102.2 to 104.4. The control remained normal. On postexposure day 17, two days after intravenous injection of the cell suspension, hemoglobinuria was observed in ewes 33, 37, and 41 but not in the non-immune control (Fig. 1,2,3,4). Hemoglubinaria persisted for at least 24 hours and was associated with a drop in hemoglobin of 2.4 to 3.9 g per 100 ml of blood. Hemoglobin values stabilized and the hemoglobinuria became less marked. The control ewe developed detectable leptospiral antibodies 48 hours after receiving the cell suspension. Reinoculation with 3 ml of the cell suspension on day 19 was followed by hemoglobinuria in ewes 33, 37 and control. Hemoglobinuria was transient in ewe 33, less than 12 hours duration, with a concurrent drop in hemoglobin of 1.4 g. Ewe 37 had marked hemoglobinuria for 36 hours accompanied by a loss of 3.1 g of hemoglobin. Hemoglobinuria was first observed in the control 48 hours after the second exposure to the cell suspension with a loss of 4 g of hemoglobin per 100 ml in a 96 hour period. Ewe 41 received one half the amount of cell suspension on reinoculation and no hemoglobinuria resulted. The agglutination-lysis titer decreased following inoculation with the cell suspension in those instances where

hemoglobinuria was observed.

Discussion. Hemoglobinemia, frequently accompanied by hemoglobinuria, is a frequent manifestation of experimental *L. pomona* infections in sheep and cattle. The pathogenicity of *L. pomona* has been attributed to the cell-free supernatant fluids of cultures. In referring to the hemolytic factor described by Alexander *et. al.*(4) Alston and Broom(12) state, "The authors suggested that the presence in leptospires of a soluble hemolytic agent may be a significant factor in determining the pathogenicity of leptospiral infections. This is a tempting speculation, but the evidence at present available does not appear to favour it." Morse *et. al.* (3) reported hemoglobinuria in 5 experimentally infected lambs. Antibodies were detected in the serum of 4 of these lambs prior to or concurrent with onset of hemoglobinuria and while circulating antibodies were present. The premise was established that, with large numbers of leptospirae present in the blood, antibody actively lyses the leptospirae releasing a hemolytic endotoxin capable of lysing red cells in presence of specific antibody and complement.

The hemoglobinuria observed in this experiment appeared to be an antibody requiring phenomenon. The control ewe did not evidence hemoglobinuria or marked hemoglobinemia on the first exposure to 3 ml of the cell suspension. A second administration of cell suspension subsequent to the appearance of circulating antibodies resulted in hemoglobinuria, suggesting that specific antibodies were required. Hemoglobinuria was usually associated with a drop in serum titer of the magnitude of one log, 24 hours after administration of the cell suspension. The drop in titer could be attributed to introduction of the specific antigen with an antibody-antigen reaction following, essentially an *in vivo* absorption phenomenon. The requirement for circulating antibody to produce hemolysis gives credence to the premise of a specific requirement for antibody plus a component of the cell suspension. Antiglobulin tests should be applied to determine the relationships between the components of the system.

Amount of cell suspension administered was directly related to degree of hemolysis observed. The ewe in Trial 1 had severe hemoglobinemia and hemoglobinuria. A second administration of 4½ ml of cell suspension injected during hemoglobinuria resulted in continued hemolysis and eventual death, attributed to the severe anemia. In Trial 2, 3 ml of cell suspension produced hemoglobinuria in immune animals while 1½ ml did not. Hemoglobin is a threshold substance and hemoglobinuria does not occur until plasma concentration reaches 150 mg%(13). Administration of 1½ ml of cell suspension did not result in hemolysis of a magnitude that would produce such a plasma concentration.

The results of this experiment suggest an antibody requiring cellular hemolytic factor, probably an endotoxin, that manifests itself in a manner that closely parallels that observed in natural or experimental infection with *L. pomona.* The unidentified factor in the cell suspension has a greater propensity as a virulence factor involved in the pathogenicity of *L. pomona* infection than the factor demonstrated in the cell-free culture supernatant fluids.

Summary. Hemoglobinuria and hemoglobinemia in ewes followed intravenous injection of disrupted *L. pomona cells.* Hemolysis occurred only in presence of antibody. It is postulated that the hemolytic effect is due to interaction of a cellular component, antibody and red blood cells.

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Effect of Gamma Irradiation and AET on Rat Blood Cholinesterase.* (26416)

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Previous work by Sabin(1) has indicated that red blood cell cholinesterase levels of the mouse offer a sensitive biological index to x-irradiation. Williams(2), by a different technic, has shown similar action of gamma irradiation in whole blood cholinesterase of the rat, but the threshold by this index was not determined in this species. In the same study the acute toxicity of acetylcholine was shown to be greater in gamma irradiated mice than in the non-irradiated animals.

With the discovery of Patt, *et al.*(3), that cysteine and glutathion gave radiation protection, further studies by Bach and Herve (4) on radio-protective agents and later work by Shapira, *et al.*(5) led to development of several radio-protective materials. One of these, S, 2-aminoethylisothiouronium bromide, hydrobromide (AET) has been studied rather extensively and was utilized in this investigation.

The purpose of this study was 2-fold: 1) to determine the irradiation threshold for whole blood cholinesterase activity in the rat and to plot a dose response curve of these data; 2) to determine whether AET produces any *in vivo* gamma irradiation protective action insofar as whole blood cholinesterase activity is concerned.

Methods. Rats utilized were irradiated by whole body exposure in lucite boxes to a cobalt-60 source of 820 curies at a source to midline distance of 150 cm. Dosimetry was accomplished utilizing the Victoreen model 70 r-meter and hi-energy chamber model 620. Exposure rate at this distance was 9.37 r per minute.

Dosage levels tested were 0, 75, 150 and 300 r except where otherwise noted. The 10 animals per level utilized were male Wistar strain Charles River rats, weighing 100 to 135 g at beginning of experiment. Cholinesterase determinations were by a micro technique similar to that of Michel(6) and involved the use of one-tenth ml of whole blood. The blood was drawn by tail vein into a one-tenth ml pipette and transferred to a 15 ml centrifuge tube containing 2 ml of distilled water as a hemolyzing agent. The fluid level in the tube was then brought up to 2.5 ml with distilled water and then to 5 ml with a buffer containing 0.82 g sodium diethyl barbiturate, 0.10 g of potassium (monobasic) phosphate and 44.70 g of potassium chloride per liter. The pH of this buffer was adjusted to 8.3 with 0.10 M hydrochloric acid, prior to use. The centrifuge tubes were then placed in a water bath at 37° ± 0.5°C and 0.5 ml of 3 per cent acetylcholine bromide was added.

The initial pH was then read and incubation continued for 2 hours after which the final pH was recorded. The difference between these values was recorded as the delta pH. Non-enzymatic hydrolysis of the acetylcholine was virtually non-existent so no correction was made. Mean initial control whole blood cholinesterase activities, i.e. delta pH, under these conditions were 1.04 pH units per 2-hour incubation period.

All animals used were given Purina Lab Chow and water *ad libitum* and were housed

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TABLE I. Mean Delta pH and Standard Error.

Group	Pre-AET—Pre-irrad.	Days after irradiation 3rd day	7th day	10th day	14th day
Control	.99 ± .028	1.08 ± .01	1.15 ± .003	1.13 ± .003	1.13 ± .02
AET control	1.03 ± .02	1.01 ± .024	1.16 ± .02	1.17 ± .02	1.06 ± .013
75 r	1.05 ± .04	1.13 ± .04	1.11 ± .03	.97 ± .02	1.06 ± .02
150 r	1.00 ± .02	1.06 ± .045	.93 ± .05	.89 ± .05	1.11 ± .07
300 r	1.11 ± .02	1.13 ± .03	.98 ± .02	.86 ± .02	.97 ± .02
AET + 300 r	1.01 ± .01	1.09 ± .02	.89 ± .02	.85 ± .03	.98 ± .004
" + 600 r	1.09 ± .02	1.11 ± .04	.81 ± .01	.82 ± .04	1.02 ± .04

5 to a cage both before and after irradiation. Cholinesterase determinations were made on all animals prior to irradiation, on the 3rd, 7th, 10th and 14th post-irradiation days.

A second series of animals of 10 per level was treated with AET intraperitoneally at 200 mg/kg 15 minutes prior to and 100 mg/kg at time of irradiation according to the method of Hanna(7).

Radiation levels on these animals were 0, 0 plus AET, 300 r plus AET and 600 r plus AET. Cholinesterase determinations were identical with those previously described and occurred before, on the 3rd, 7th, 10th and 14th days after irradiation. All statistical treatment is by students "t" test.

Results. Results obtained in various phases of this study are incorporated into Table I. These values present the mean delta pH and standard error of blood samples taken from beginning groups of 10 animals in each of the treatment groups except control where 20 were utilized.

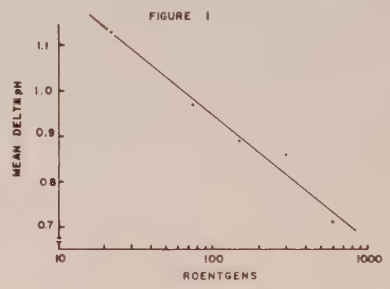
Statistical comparisons of the various groups are found in Table II. It is apparent that peak significant post-irradiation depression of whole blood cholinesterase activity occurs on the tenth day.

TABLE II. Percent Variation in Mean Delta pH from Control.

	Pre-irrad.	Day after irradiation 3rd	7th	10th	14th
300 r	+12.0*	+4.6	−14.8*	−23.8*	−14.2*
150 r	+ 1.0	− 2.0	−19.1*	−21.2*	− 1.8
75 r	+ 6.0	+4.6	− 3.5	−14.1*	− 4.4
AET control	+ 4.0	− 6.4*	+ .8	+3.5	− 4.4*
Percent variation in mean delta pH when 300 r + AET is compared with 300 r alone					
300 r	+9.9*	+3.7	+10.1*	+1.2	−1.0

* P <.05 by Student's "t" test.

It is possible from these data to construct a dose response curve based upon whole blood cholinesterase activity on the tenth day after gamma irradiation (Fig. 1). By adding a 600 r point from our previous study (2) and by extrapolating the line drawn between these points, with the control delta pH on the ordinate, it is possible to estimate the approximate threshold for irradiation which would be necessary to produce just measurable change in whole blood cholinesterase. This extrapolation indicates that the control level falls between 20 and 30 r. Thus, we



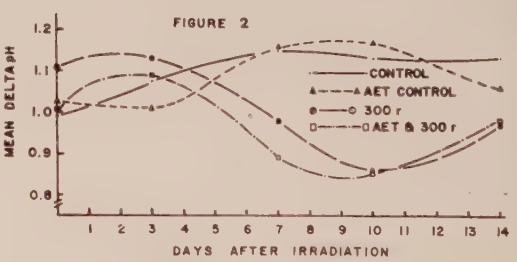


FIG. 1. Radiation levels plotted against the mean delta pH for rat whole blood cholinesterase on tenth day after irradiation.

FIG. 2. Mean whole blood cholinesterase levels (delta pH) at 0, 3, 7, 10 and 14 days after irradiation.

could expect just measurable changes within this range if the phenomenon fits a semi-log plot and if sufficient numbers of animals were used.

Fig. 2 presents data obtained from Table I with specific regard to AET. The upper 2 curves present time-mean delta pH plot of the 2 controls (i.e. with and without AET), while the lower present 300 r, with and without AET (Table II).

Discussion. The finding that apparent threshold levels of gamma irradiation in the rat are in the vicinity of 20 to 30 r for whole blood cholinesterase is in complete agreement with Sabin(1) who found similar activity with x-irradiation down to 25 r in the mouse red cell cholinesterase. Thus the two species are remarkably similar despite differences in type of irradiation and different enzyme entities.

The fact that AET fails to protect whole blood cholinesterase from gamma irradiation is evident from Fig. 2 as the 2 levels are virtually identical, i.e. 1.2% apart, on the tenth day, the time of expected peak depression. AET at the high levels tested did not appreciably affect cholinesterase in the surviving animals, though mild significant depression attributable to AET alone was noted on the third and fourteenth days (Table II).

Summary. 1. Whole body gamma irradiation in the rat produced significant whole blood cholinesterase depression on the tenth day at a dosage level of 75 r. 2. The levels tested when plotted and extrapolated indicated threshold changes in cholinesterase activity would be in the vicinity of 20 to 30 r. 3. AET alone, while producing some mild cholinesterase depression, failed to protect whole blood cholinesterase activity from the effects of gamma irradiation at the levels of agent and irradiation tested.

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Effects of Source of Dietary Carbohydrate on Survival Time of Sublethally X-irradiated Mice.* (26417)

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During an investigation of the effects of diet on response of mice exposed to multiple sublethal doses of total body x-irradiation, it was observed that length of survival of x-irradiated animals was significantly affected by the source of dietary carbohydrate employed. Our findings are reported here.

Procedure. The basal ration employed consisted of carbohydrate, 59%; casein†, 24%; cottonseed oil, 10%; salt mixture‡, 5%; cellulose§, 2%; and the following vitamins per kg of diet: thiamine hydrochloride, 10 mg; riboflavin, 10 mg; pyridoxine hydrochloride, 10 mg; calcium pantothenate, 60 mg; nicotinic acid, 100 mg; ascorbic acid, 200 mg; biotin, 4 mg; folic acid, 10 mg; para-aminobenzoic acid, 400 mg; inositol, 800

* This investigation was supported in part by Medical Research contract with the Surgeon General's Office, Dept. of Army, Washington, D. C.

† Vitamin-free Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

‡ Wesson Modification of Osborne-Mendel Salt Mixture, General Biochemicals, Inc., Chagrin Falls, Ohio.

§ Solka Floc, Brown and Co., Boston, Mass.

TABLE I. Effects of Source of Dietary Carbohydrate on Survival Time of Mice Exposed to Multiple Sublethal Doses of Total Body X-irradiation.*

Dietary carbohydrate	No. of animals	Avg body wt at time of 1st x-irrad. (g)	Day after 1st x-irrad. when 50% of animals in group were dead	No. surviving†	Avg length of survival after 1st x-irrad. (days)‡§
Glucose	29	31.8	66	8	73.3 ± 6.6
Sucrose	29	32.9	55	1	54.3 ± 3.8
Dextrin	29	30.0	44	1	45.6 ± 3.3
Cornstarch	26	30.1	34	2	41.8 ± 4.8

* Each group initially consisted of 30 mice. Animals that died before 4th x-irradiation were not included in tabulation of data. Dextrin employed (Dextrin White from corn) was obtained from Mallinkrodt Chemical Works, St. Louis, Mo.; cornstarch from Mefford Chemical Co., Los Angeles, Calif.

† Experiment was terminated 118 days after 1st x-irradiation.

‡ Data were calculated on basis of an avg survival time of 118 days for animals alive at termination of experiment.

§ Including stand. error of mean.

mg; vit B_{12}, 150 μg; 2-methyl-1,4-naphthoquinone, 5 mg; choline chloride, 2 g; vit A, 5000 U.S.P. units; vit D_2, 500 U.S.P. units; and alphatocopherol acetate, 100 mg. The vitamins were added in place of an equal amount of dietary carbohydrate. Four carbohydrates were employed, diet A, glucose; diet B, sucrose; diet C, dextrin; and diet D, cornstarch.

One hundred and sixty male mice of the Webster strain were selected at 11 to 14 g in body weight and were divided into 4 comparable groups consisting of 40 animals per group. These were placed in metal cages with raised screen bottoms (5 animals per cage) and were fed the various diets indicated above. Food and water were provided *ad libitum*. The animals were fed daily and all food not consumed 24 hours after feeding was discarded. After 6 weeks of feeding 10 of the mice in each dietary group were selected at random to serve as non-irradiated controls. The remaining mice received an exposure of 200 r total body x-irradiation, repeated once weekly until a total dose of 1200 r (6 exposures) had been administered. Animals were continued on their respective diets for 118 days after first x-ray exposure or until death, whichever occurred sooner. All mice that died before the 4th x-ray exposure were not included in tabulation of data. Data were obtained as to the day after the first x-ray exposure on which 50% of the animals in each group were dead and average length of survival on each of the diets employed.

The following radiation factors were employed: GE Model Maximar 250; 250 kv; 15 ma; 0.5 mm Cu and 1 mm Al filters plus a Cu parabolic filter‖; HVL, 2.15 mm Cu; target distance to top of box, 82 cm; and dose rate, 15.6 r per minute (measured in air, at top of box). The animals to be irradiated were placed in a wooden box divided into 60 equal compartments 1¼″ wide, 3″ long, and 1½″ deep. The partitions and top were made of ⅛″ cellulose acetate sheeting; and the top and bottom of each compartment were perforated with holes for purposes of ventilation. The container was rotated slowly on an electrically driven turntable to insure equivalent irradiation.

Results. Significant differences were observed between the various dietary groups as to the day on which 50% mortality occurred and average length of survival following exposure to multiple sublethal doses of total body x-irradiation (Table 1). Survival time was least for the mice fed dextrin and cornstarch (diets C and D), slightly longer for those fed sucrose (diet B) and significantly longer for those fed glucose as the source of dietary carbohydrate. The increased survival on the latter diet occurred despite the

‖ A nonuniform filter which produces a flat isodose surface of x-ray intensity constructed by the method of Greenfield and Hand(7). The center of the filter had a thickness of 1.7 mm Cu; the edge, 0.5 mm Cu.

fact that mice in this group received more total radiation than those in the other groups. This situation obtained because irradiation was continued as the experiment progressed, hence the mice which survived were exposed to a greater total dose of x-irradiation than those which succumbed earlier in the period. No significant differences in survival were observed among non-irradiated mice in the various groups with at least 80% of the animals in all dietary groups surviving the experimental period of 160 (42 + 118) days.

No data are available as to the cause of the diverse results obtained with the different carbohydrates. It is well established that the intestinal epithelium is highly sensitive to x-irradiation (1-6), and it is possible that multiple sublethal doses of total body x-irradiation induced changes in respect to digestion and absorption of carbohydrates that resulted in a simple sugar such as glucose being utilized more efficiently and with less toxic effects than was the case when more complex carbohydrates were fed.

Summary. Experiments were conducted to determine the effects of source of dietary carbohydrate on survival time of mice exposed to multiple sublethal doses of total body x-irradiation. Findings indicate that average survival time of x-irradiated mice was significantly longer on a glucose-containing diet than on rations containing sucrose, dextrin or cornstarch as the source of dietary carbohydrate.

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A Colorimetric Method for Assay of Erythrocytic Glucose-6-phosphate Dehydrogenase.* (26418)

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A disorder known as primaquine-sensitive hemolytic anemia(1,2) in humans is proving to be increasingly useful as a tool for study of genetic homeostasis(3) and biochemical genetics(4,5,6). This disorder was first reported by Carson(7) to be associated with a deficiency in activity of glucose-6-phosphate dehydrogenase in red blood cells. More recently, 2 different enzymic expressions of the deficiency have been uncovered(8). Investigations of these disorders have been handicapped by the necessity, when the enzyme is to be measured accurately in hemolysates, for a spectrophotometer capable of operation in the ultraviolet range and equipped with a photomultiplier to compensate for the very high optical density of the hemolysate-blank. Motulsky and Campbell(9) have adapted the technic of Dickens and Glock (10) to permit useful visual estimation of the activity of erythrocytic glucose-6-phosphate dehydrogenase in genetic field work. There would seem to be a need for an objective assay requiring only relatively commonplace equipment.

A method of assaying human erythrocytic

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glucose-6-phosphate dehydrogenase has been found which preserves all of the accuracy of the previous assay as well as much of the simplicity of the method of Motulsky and Campbell. The only instrument required is a simple spectrophotometer or photoelectric colorimeter such as can be found in many clinical or chemical laboratories. In this method, which is adapted from a general assay for dehydrogenases(11), phenazine methosulfate (PMS) is used as an electron carrier between reduced triphosphopyridine nucleotide (TPNH) formed in the reaction and dichloroindophenol (DCIP). Rate of reduction of DCIP is followed at 620 mμ. At this wave length absorption of DCIP is nearly maximal, while the erythrocyte hemolysate has an absorption about 2% of that of the DCIP at the concentration used in the assay.

Method. The reaction mixture contains 1.0 ml of 0.5 M tris buffer, pH 7.5, 0.1 ml of 1 M $MgCl_2$, 0.4 ml of 0.01% Na 2,6-dichloroindophenol (these reactants may be mixed and stored as a stock solution in the refrigerator, provided the solution is allowed to warm to room temperature before use), 0.1 ml of 0.01 M triphosphopyridine nucleotide (TPN), 0.05-0.1 ml of a 1/20 stroma-free erythrocyte hemolysate(12), 0.5 ml of 0.05 mg/ml phenazine methosulfate, 0.5 ml of 0.02 M Na glucose-6-phosphate, pH 7.5 (because of the relatively low Michaelis constant for glucose-6-phosphate(13), the use of 0.2 ml is permissible) and water to make a total volume of 6.0 ml. TPN and glucose-6-phosphate solutions are stored frozen. The PMS solution must be protected from light and is preferably stored in the cold. All reactants except glucose-6-phosphate are added to the cuvette, which is then left at room temperature for 10 minutes. Omission of this preincubation period, during which easily oxidizable substances in the hemolysate react with DCIP, results in non-linearity of the measurement. This is an additive effect of the enzymic reaction and a non-linear, non-enzymic reduction of DCIP. After the preincubation period, glucose-6-phosphate is added to the cuvette and reduction of DCIP is followed at 620 mμ, using a water blank to zero the colorimeter. Readings are taken at 30 or 60 second intervals. The rate remains linear for 10 minutes and is proportional to the amount of hemolysate added within the limits 0.02 to 0.2 ml. A battery-operated Coleman, Jr. spectrophotometer was employed in this study. Periodic checks showed that this instrument was free enough from drift so that the initial zeroing sufficed for the entire measurement period.

The assays reported here were run at room temperature. The amount of PMS specified, which is lower than that used previously(11), gives a maximum reaction rate with glucose-6-phosphate dehydrogenase. Higher concentrations are inhibitory. The pH employed in the present assay is below the pH optimum of this enzyme as measured by other methods(8). However, with PMS as electron carrier, the reaction rate is greater at pH values close to 7.5 than at higher pH values. Activity of the enzyme may be expressed as mOD units change in absorbancy per minute per ml of hemolysate or as mμmoles of DCIP reduced per minute per ml of hemolysate.

Results and discussion. To test the method, activities of erythrocyte hemolysates from a normal and from a glucose-6-phosphate dehydrogenase deficient individual as well as mixtures of the 2 hemolysates were determined by the present method (PMS method) and by the adaptation of Zinkham *et al.*(12) of the method of Kornberg and Horecker(14) (UV method). Assays by the latter method were carried out at pH 8.0. The cuvette temperature on the day these assays were performed was about 33°C in the UV method and 25-27° in the PMS method. The results are shown in Fig. 1. Rates determined by each method are proportional to amount of enzyme present. The lower activity by the PMS method is due in part to the lower pH at which the PMS assay is run and, in this experiment in which temperature equilization was not attempted, to the temperature difference. When measurements were made at the same temperature by the two methods, and corrected for the increased activity at pH 8.0(8), turnover rates by the 2 methods were approximately equal. In the

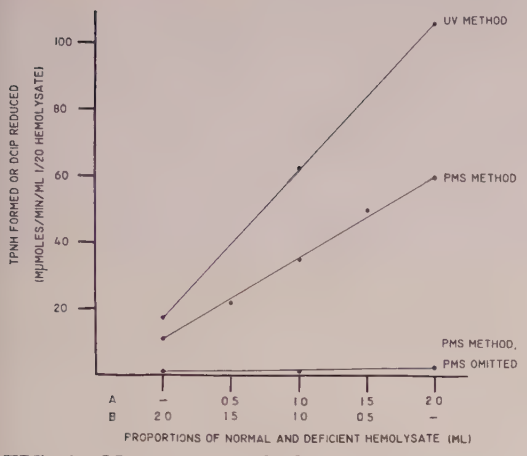

FIG. 1. Measurement of glucose-6-phosphate dehydrogenase activity of normal and deficient hemolysates and mixtures of the two by PMS method and UV method. Values for DCIP reduction are calculated from an observed molar extinction coefficient of 17.6×10^3 for DCIP in 0.083M tris, pH 7.5.

A. Hemolysate from normal subject.
B. " " deficient subject.

PMS assay there is essentially no activity when PMS is omitted, indicating that in these stroma-free hemolysates diaphorase activity capable of coupling TPNH and DCIP is absent.

In addition to the advantage of requiring only readily available equipment, the present method makes it possible, because of the higher extinction coefficient of DCIP at 620 mμ than of TPNH at 340 mμ and because of the lower absorbancy of hemoglobin at the former wave length, to measure lower activities of the dehydrogenase in hemolysates than has hitherto been possible. The ratio

$$\frac{\triangle \text{O.D.}_{\text{PMS METHOD}} / \text{O.D. of hemolysate at 620 m}\mu}{\triangle \text{O.D.}_{\text{UV METHOD}} / \text{O.D. of hemolysate at 340 m}\mu}$$

is equal to approximately 110. The question of the necessity of a diaphorase, which arises in a colorimetric determination of dehydrogenase activity, is obviated since the present method is independent of a diaphorase activity. A few experiments have been done which suggest that this method should be readily adaptable to visual estimation of erythrocytic glucose-6-phosphate dehydrogenase.

Summary. A colorimetric method for estimation of glucose-6-phosphate dehydrogenase activity of erythrocytic hemolysates is described. The method reported employs phenazine methosulfate as an electron carrier between TPNH formed in the reaction and dichloroindophenol. Rate of reduction of the latter compound is followed spectrophotometrically. The method gives turnover rates comparable to those obtained with the ultraviolet spectrophotometric method and is more sensitive than the latter. The assay utilizes a standard spectrophotometer or photoelectric colorimeter and is independent of diaphorase activity. The method should be readily adaptable to visual estimation of glucose-6-phosphate dehydrogenase of erythrocytes.

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Mycobacterium tuberculosis H37Rv Grown *in vivo:* Nature of the Inhibitor of Lactic Dehydrogenase of *Mycobacterium phlei.* (26419)

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Previous reports from this laboratory(1, 2,3,4) have shown that cells of *Mycobacterium tuberculosis* H37Rv grown *in vivo* (LH37Rv) adsorb from lung tissue of tuberculous mice a substance which inhibits electron transport system in cell-free extracts of *Mycobacterium tuberculosis* H37Rv, *Mycobacterium tuberculosis* BCG and *Mycobacterium phlei* as well as in lung homogenates of normal mice. Cell-free extracts of *M. phlei* were subsequently used as an indicator system for detection of the above inhibitor. It has been found that extracts from various organs of both infected and normal mice displayed an inhibitory activity similar to that of LH37Rv cells, this activity being much more pronounced in organs of tuberculous mice. To ascertain whether the inhibition brought about by extracts from the various organs of normal and infected mice and by LH37Rv cells was due to the same substance, study to elucidate the nature of the inhibitor was undertaken. This is reported here.

Materials and methods. The organisms used were: *Mycobacterium tuberculosis* LH37Rv, *Mycobacterium tuberculosis* H37 Rv and *Mycobacterium phlei.*

M. tuberculosis H37Rv and *M. phlei* were grown in media previously described(2). *M. tuberculosis* LH37Rv was obtained from lungs of moribund mice infected with *M. tuberculosis* H37Rv. Tubercle bacilli were separated from lungs by a procedure described elsewhere(2). For experiments on inhibition of lactic dehydrogenase of *M. phlei,* the tubercle bacilli grown *in vivo* after separation from lungs were washed twice in a 0.1 M phosphate buffer pH 7.2 and resuspended in the same buffer to a density corresponding to 3-4 mg dry weight per 1 ml. Cells of *M. phlei* were harvested by centrifugation, washed twice in a 0.1 M phosphate buffer pH 7.2 and resuspended in the same buffer, 50 mg dry weight of cells per 1 ml of buffer. The resulting thick suspension was disintegrated for 45 minutes in a 9 KC Raytheon magnetostriction oscillator. Cell debris and unbroken cells were removed by centrifugation at 9,000 rpm in an MSE high speed centrifuge in the cold. The cell-free supernatant containing 12 to 15 mg protein per 1 ml was used throughout. Protein content was estimated by the biuret method. Cell-free extracts of *M. phlei* are occasionally referred to as enzyme preparation. When required, the enzyme preparations were dialyzed in the cold for 24 hours against frequent changes of distilled water. Lactic dehydrogenase activity of cell-free extracts of *M. phlei* was determined by reduction of triphenyltetrazolium chloride under aerobic conditions. Reaction mixture contained: 0.5 ml of enzyme preparation; 0.5 ml of 0.1 M phosphate buffer pH 7.2 (or 0.5 ml of various additions in the same buffer); 0.1 ml of 0.3 M sodium lactate and 0.1 ml of 1% solution of triphenyltetrazolium chloride. Time of reaction was 10 minutes, temperature 37°. The reaction was arrested by addition of 2 ml of *iso*-butanol, formazan was extracted twice with the same solvent and its color intensity read in a Coleman Jr. spectrophotometer at 485 mμ. For preparation of extracts from organs of mice, the various organs were homogenized in a teflon homogenizer run at top speed for 2 minutes, and centrifuged at 7,200 × g for 10 minutes in the cold. The supernatant was used throughout. All procedures were carried out in the cold; 4% homogenates were used. The extracts can be stored for weeks at −10° without losing their inhibitory activity.

DPNase (Pyridine transglycosidase) was assayed by the method of Kaplan *et al.*(5), based on the cyanide reaction of diphosphopyridine nucleotide (DPN). Reaction mixture contained: 0.4 parts of enzyme; 0.4

TABLE I. Some Physical Properties of Inhibitor of Lactic Dehydrogenase of *M. phlei.*

Previous treatment of preparations containing inhibitor	Lactic dehydrogenase of cell-free extracts of *M. phlei* (O.D. of formazan) in presence of			
	LH37Rv cells	Lung extracts	Spleen extracts	Control
Non-treated	.28	.60	.40	2.80
Heated at 80° for 5 min.	2.85	2.70	2.70	
Dialyzed	.16	.48	.40	

For experimental conditions see *Materials and methods.*

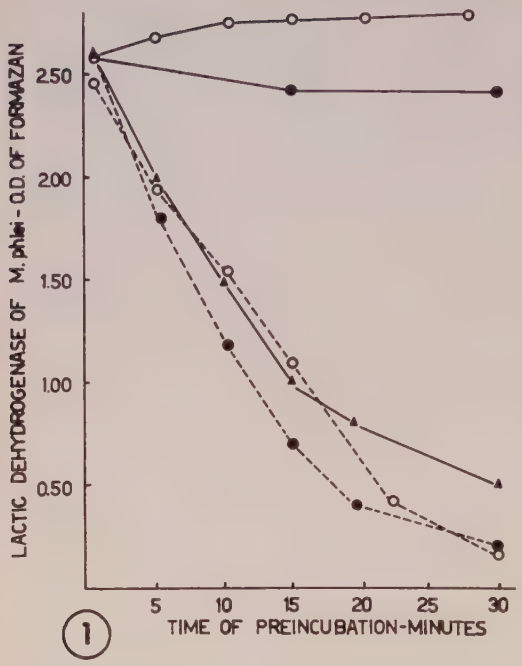
parts of 0.1 M phosphate buffer pH 7.2 and 0.2 parts of 0.003 M DPN (purchased from N. B. Co., Cleveland, Ohio). Before and after various time intervals of incubation at 37°, 1 ml samples were withdrawn, 3 ml of 1.0 M KCN were added and the mixture read at 325 mμ in an Unicam spectrophotometer. Samples with KCN without DPN served as blanks. When LH37Rv cells were used as the source of the enzyme, the cells were removed by centrifugation after addition of KCN.

Results. Some physical properties of the inhibitor. The inhibitor of the electron transport system of *M. phlei,* found in the extracts from various organs of mice and on the surface of *M. tuberculosis* H37Rv grown *in vivo* is thermolabile and nondialyzable. Heating of LH37Rv cells, lung or spleen extracts at 80° for 3 to 5 minutes completely destroyed their inhibitory activity. Dialysis, on the other hand, did not affect their inhibitory activity (Table I).

Kinetics of inhibition. It has been previously shown(3) that rate of inactivation of lactic dehydrogenase of *M. phlei* by the various preparations containing the inhibitor depends on concentration of the inhibitor in reaction mixture. Inactivation of lactic dehydrogenase of *M. phlei* by concentrated preparations of the inhibitor was apparent immediately after addition of the latter. On the other hand, diluted preparations of the inhibitor inactivated lactic dehydrogenase of *M. phlei* only after preincubation.

To study in detail the kinetics of inhibition we have used LH37Rv cells, lung or spleen extracts (from either normal or tuberculous mice) in such amounts as did not inhibit lactic dehydrogenase of the enzyme preparations when added to the reaction mixture simultaneously with lactate and tetrazolium. The various preparations containing the inhibitor were incubated with the enzyme preparation at 37° and 4°, at various time intervals samples were withdrawn, lactate and tetrazolium were added and lactic dehydrogenase of the enzyme preparations was determined. The results are illustrated in Fig. 1. Incubation of lung and spleen extracts and of LH37Rv cells with the enzyme preparation caused a gradual decrease in lactic dehydrogenase activity of the latter. The decrease in enzyme activity was only apparent on incubation at 37°. Incubation of the enzyme preparation with the inhibitor at 4° left lactic dehydrogenase activity of *M. phlei* unimpaired.

Restoration of dehydrogenase activity in inactivated extracts of M. phlei. Results of the above experiments suggested that inhibition of lactic dehydrogenase in cell-free extracts of *M. phlei* was caused by destruction of some component of lactic dehydrogenase system of the above organism. To prove this, we have performed experiments on restoration of dehydrogenase activity in the inactivated enzyme preparation. These experiments were carried out as follows: Lactic dehydrogenase in cell-free extracts of *M. phlei* was inactivated by incubation of the enzyme preparation with LH37Rv cells, spleen or lung extracts. When LH37Rv cells were used they were removed by centrifugation from the enzyme preparation after inactivation of the enzyme was completed. Dialysed or boiled extracts of non-treated *M. phlei* were added to the inactivated enzyme preparation and the effect of these additions on lactic dehydrogenase activity of the latter was determined. The results of these experiments (Table II) clearly show that addition of boiled extracts from non-treated *M. phlei*



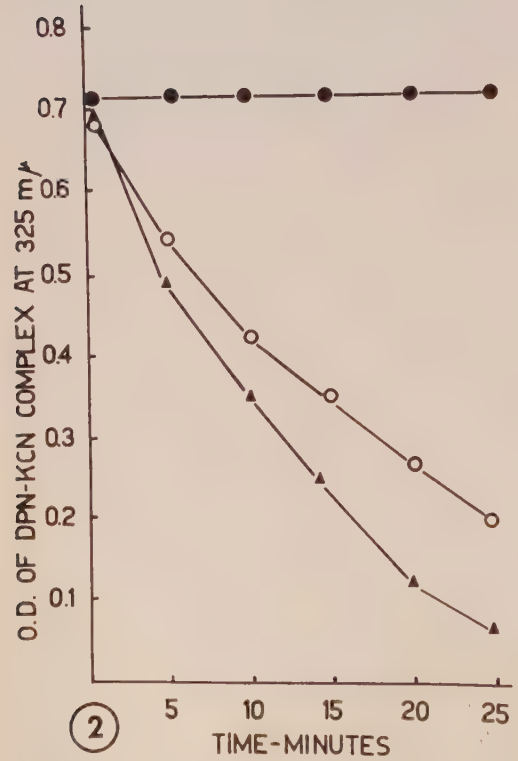


FIG. 1. Effect of preincubation of inhibitor with cell-free extracts of *M. phlei* on lactic dehydrogenase activity of the latter. Reaction mixture contained: 0.5 ml of cell-free extracts of *M. phlei*; 0.5 ml of lung extract (when present); 0.2 ml of spleen extract (when present); 0.5 ml of LH37Rv cells (when present). After preincubation for time indicated in Fig., 0.1 ml of 0.3 M sodium lactate and 0.1 ml of 1% solution of triphenyltetrazolium chloride were added. Formazan was measured after 10 min. incubation at 37°.

○– – –○ Lactic dehydrogenase activity of cell-free extracts of *M. phlei* preincubated at 37° with LH37Rv cells.
▲——▲ *Idem* with lung extract.
●– – –● " " spleen " .
○——○ " , non-treated (control).
●——● Lactic dehydrogenase activity of cell-free extracts of *M. phlei* preincubated with LH37Rv cells at 4°.

Since preincubation of cell-free extracts of *M. phlei* with lung or spleen extracts at 4° gave identical curves to that of preincubated extracts with LH37Rv cells, they are omitted from figure.

FIG. 2. DPNase activity of H37Rv cells, LH37Rv cells, and spleen extracts of mice. For experimental conditions see *Materials and methods.*

○——○ LH37Rv cells
●——● H37Rv "
▲——▲ Spleen extract

completely restored dehydrogenase activity of the inactivated enzyme preparation. On the other hand, addition of dialyzed extracts of *M. phlei* failed to restore the enzymatic activity in inactivated extracts of *M. phlei.* It should be pointed out that the various additions to the enzyme preparation inactivated by spleen or lung extracts were made in presence of these extracts. This was permissible since the amount of inhibitor used was so small that it did not inhibit the lactic dehydrogenase of the enzyme preparations without preincubation. It was then shown that boiled extracts of non-treated *M. phlei* lost their restorative property after incubation with the various preparations containing the inhibitory substance. Addition of boiled extracts so treated, to the inactivated enzyme preparation was done after destroying the inhibitor by boiling the reaction mixture for 5 minutes. This was permissible since the factor inactivated by the inhibitor was thermostable.

Having ascertained that the inhibitory effect of LH37Rv cells, lung or spleen extracts of mice, both normal and infected, was caused by inactivation of some heat stable component of lactic dehydrogenase system of *M. phlei,* we determined the effect of known coenzymes of lactic dehydrogenase on

TABLE II. Restoration of Lactic Dehydrogenase Activity in Cell-Free Extracts of *M. phlei* Inactivated with LH37Rv Cells, Lung or Spleen Extracts.

Additions	Lactic dehydrogenase of cell-free extracts of *M. phlei* (in O.D. of formazan) inactivated by			
	LH37Rv cells	Lung extract	Spleen extract	Control of non-treated extracts of *M. phlei*
No additions	.51	.46	.21	3.10
Dialyzed extracts of non-treated *M. phlei*	.61	.58	.18	5.10
Boiled extracts of non-treated *M. phlei*	3.20	3.14	3.21	3.51
Idem after incubation with inhibitor	.32	.48	.16	3.10
DPN	3.20	3.10	3.40	3.40
DPN after incubation with inhibitor	.34	.50	.16	3.10

Lactic dehydrogenase of *M. phlei* was inactivated by procedure described in text and Fig. 1. For lactic dehydrogenase assay see *Materials and methods.*

restoration of enzymatic activity in the inactivated enzyme preparations. Addition of diphosphopyridine nucleotide to extracts of *M. phlei* inactivated by LH37Rv cells, lung or spleen extracts completely restored their dehydrogenase activity (Table II). Moreover, it has been shown that DPN after incubation with LH37Rv cells, spleen or lung extracts completely lost its restorative property.

Nature of the inhibitor. Since destruction of DPN may be accomplished by the action of one of 2 enzymes: the nucleotide pyrophosphatase or pyridine transglycosidase (DPNase), we performed experiments to determine which enzyme is operative in this case. For this purpose LH37Rv cells as well as lung or spleen extracts were incubated with DPN and hydrolysis of DPN was followed spectrophotometrically by determination of DPN level by cyanide. The results (Fig. 2) show clearly that DPN was hydrolyzed at the nicotinamide ribose linkage. It has therefore been concluded that the inhibition of lactic dehydrogenase of *M. phlei* brought about by LH37Rv cells, lung and spleen extracts of mice is due to cleavage of DPN by DPNase present in all these preparations.

Discussion. Discussing the possible role of the inhibitor adsorbed on tubercle bacilli grown *in vivo* in the pathogenesis of tuberculosis we have assumed(3) that the tubercle bacilli upon ingestion by phagocytes introduce the inhibitory substance into the phagocytizing cell. The result is inhibition of phagocyte respiration and eventually its death. Results presented here show that the inhibitor is DPNase. Enzymes destroying DPN have been found in plague toxin(6) and in streptococcal culture supernates(7). A remarkable correlation between leukotoxicity and capacity of streptococci to produce DPNase was observed(7). The authors assumed that leukotoxicity may be due to destruction of DPN of phagocytes by DPNase. If these assumptions are proved to be true, then *M. tuberculosis* H37Rv grown *in vivo* will provide an interesting example of a microorganism which employs host enzyme for destruction of host cell. The effect of *M. tuberculosis* H37Rv grown *in vivo* on phagocytes and the role of DPNase in destruction of the latter is being investigated.

Summary. Some physical properties and kinetics of the action of the inhibitor of lactic dehydrogenase of *M. phlei,* present in spleen and lungs of mice and on the surface of *M. tuberculosis* H37Rv grown *in vivo* have been studied. The inhibitor was identified as DPNase.

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Studies of Fibroblastic Cells Cultivated from Bone Marrow of Leukemic and Non-Leukemic Patients.* (26420)

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Technics employed for *in vitro* preparation of human bone marrow cell cultures have been reviewed by Osgood(1), Reisner(2) and Berman(3). The present report describes a procedure for cultivating cells from leukemic and normal bone marrows on feeder layers of human amnion cells. Employing this technic, the susceptibility of normal and leukemic bone marrow cell cultures to certain viruses was investigated. The fluorescent antibody staining technic was also applied in attempts to detect a specific antigen in leukemic bone marrow cell cultures and efforts were made to isolate a cytopathogenic agent from leukemic marrow aspirates.

Materials and methods. Sternal bone marrow aspirates were withdrawn aseptically in 1-3 ml amounts from patients undergoing open cardiac surgery and from children with acute leukemia. The latter specimens were obtained during the course of diagnostic and follow-up aspirations performed at the Tumor Therapy Clinic of Children's Hospital Medical Center. To each ml of aspirated material was added 0.0025 mg of sterile heparin(4). Aspirates were suspended in bovine amniotic fluid (BAF) medium(5) containing 20% horse serum which had been inactivated for a half hour at 56°C. Nucleated cell counts were performed on the final suspensions.

Primary monolayer cultures of trypsinized human amnion cells (HA) were prepared in 150 × 16 mm test tubes as previously described(5). The monolayers were nourished with BAF medium containing 5% inactivated horse serum. In some experiments 5-15-day-old HA monolayers were x-irradiated with 3,500 r. They were exposed for 12 minutes and 40 seconds at a distance of 40 cm from a Siemens x-ray tube operating at 250 KV and 15 ma, filtered with 1 mm of aluminum. The dose rate was 277 r/min. X-irradiation was of sufficient intensity to arrest cellular multiplication(6). Cell viability, however, appeared to be unaffected as judged by morphologic appearance and acid production.

Viruses. The behavior of the following viruses was studied in bone marrow cell cultures:

Measles, Edmonston strain, 28th human kidney cell passage(7).

Mumps, Enders strain, 47th chick amniotic sac passage.

Sindbis, Strain AR339, 10th chick embryo cell passage(8).

Poliovirus Type I, Brunhilde strain, passed 1× in human uterine and 2× in human amnion cell cultures.

Herpes simplex, L strain, passed 5× in chick embryo amniotic sac and 3× in chick embryo cell cultures(9).

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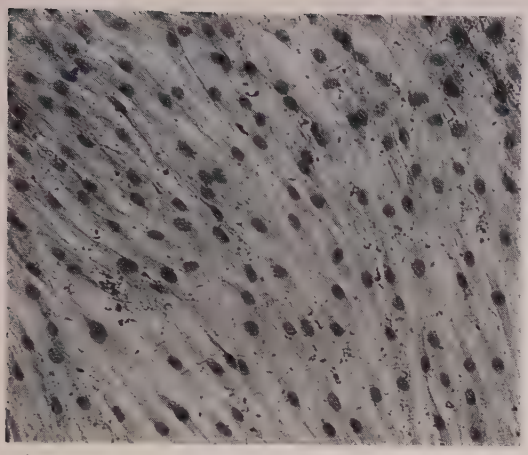

FIG. 1. Monolayer of fibroblastic cells of human bone marrow origin. The monolayer obscures underlying feeder layer of human amnion cells. H & E. × 115.

Influenza virus A/Formosa/3-13-57.

Experimental. Bone marrow cell culture technics. Bone marrow aspirates in 1-3 ml amounts were suspended directly in BAF medium containing 20% inactivated horse serum and 1 ml aliquots were dispensed into tubes containing x-irradiated and non-irradiated HA monolayers. Total nucleated cell counts of these preparations averaged 110 million cells, and each aspiration yielded an average of 60 cell culture tubes. Centrifugation of bone marrow aspirates and use of the resulting supernatant fluid and buffy coat resulted in a reduced yield of nucleated cells. Tubes were incubated in a slanting position at 37°C. Culture fluids were changed initially after 2-3 days and thereafter every 3-4 days until fibroblastic cells developed (Fig. 1). The term "fibroblastic" is used only in a descriptive sense. When confluent fibroblastic sheets became established, BAF medium containing 5% inactivated horse serum was used and tubes could be incubated at 32°C. Cellular metabolism was reduced at the lower temperature and culture fluids needed to be changed less frequently. This method of cultivating uncentrifuged bone marrow cells appeared consistently reproducible.

A total of 157 centrifuged and uncentrifuged bone marrow specimens were cultivated. Fibroblastic sheets developed from 61 (72%) aspirations obtained from 85 patients free of hematologic disorders and from 43 (60%) withdrawn from 72 leukemic subjects.

Fibroblastic growth developed equally well on x-irradiated and non-irradiated HA monolayers. The fibroblastic sheets remained in good condition when maintained at 32°C for 3-4 months. Attempts to grow bone marrow cells directly on glass were infrequently successful. Although a single cell culture of fetal bone marrow on feeder layers was subcultured 17 times, repeated efforts to establish a permanent strain of these fibroblastic cells failed.

The development of both normal and leukemic bone marrow cell cultures was characterized by 3 cytological phases similar to those described by Berman(3) and Woodliff (10). In the first phase myeloid cells were morphologically distinguishable. During the second phase large, round monocytoid cells predominated, many of which became bipolar. The third phase was marked by the appearance of sheets of fibroblastic cells which covered and obscured the underlying human amnion. The duration of the different phases varied and overlapped. Generally, sheets of fibroblastic cells formed in 10-14 days. When fibroblastic sheets did develop on glass, the same 3 phases were observed. Cells of epithelial morphology did not appear in our cultures.

Attempted differentiation between normal and leukemic bone marrow cell cultures. There were no apparent differences in morphology, rate of metabolism, or type of degeneration. The indirect fluorescent antibody technic(11) was used unsuccessfully in an effort to detect a specific antigen in fibroblastic tissue cultures derived from leukemic marrows. Leukemic serum, obtained from children in relapse and during the remissive stage of the disease, and labelled horse anti-human gamma globulin§ were consecutively layered on normal and leukemic bone mar-

§ Obtained from Sylvania Chemical Co., Orange, N. J. and adsorbed with both human and mouse liver powder to remove non-specific staining material.

row cell cultures. No specific fluorescence was observed.

Effect of viruses on bone marrow cells. Experiments were performed to determine whether there were differences in viral susceptibility between the cultures derived from normal and lukemic aspirates. Both normal and leukemic marrow cell cultures supported the propagation of poliomyelitis, measles and herpes simplex viruses. Polio and herpes simplex viruses caused cytopathic changes in the fibroblastic cells which were observable under low power microscopic inspection. Measles virus chronically infected both types of marrow cell cultures; although no cytopathic lesions were seen, typical inclusion bodies(7) were observed after hematoxylin and eosin staining. This chronic infection persisted for 4 months and was confirmed by demonstration of infectivity of the tissue culture fluids for primary human amnion cell cultures in which typical cytopathic changes were observed(5). Neither normal or leukemic marrow cultures supported the growth of egg adapted mumps virus. Only normal marrow cell cultures were inoculated with Sindbis and influenza viruses. Sindbis virus was cytopathogenic for these cultures. Influenza virus was not cytopathogenic for normal bone marrow cell cultures, but after inoculation such cultures hemadsorbed both guinea pig and chicken erythrocytes. The hemadsorption technic of Vogel and Shelokov(12) was used in these studies.

Attempts to demonstrate a transmissible agent in leukemic cells. Efforts were made to isolate a cytopathogenic agent from aspirates obtained from leukemic patients. Primary human amnion, monkey kidney (rhesus), chick embryo cell and normal bone marrow cell cultures were inoculated with untreated leukemic aspirates and with ground fibroblastic cells and supernatant fluid obtained from leukemic cell cultures. Cytopathic changes were not observed.

Discussion. The origin of the fibroblastic cell in bone marrow cell cultures remains obscure. It has not been determined whether these cells are of hemic origin or derive from the connective tissue. If hemic in origin they may represent the transformation of more than one type of bone marrow cell.

We have not determined why HA feeder layers so readily promote the development of bone marrow cells. Attempts to explore this problem were made by suspending and maintaining bone marrow aspirates in various proportions of stock BAF medium and BAF medium that had nourished HA monolayers ("conditioned" medium). However, the use of "conditioned" medium did not facilitate development of fibroblastic sheets on plain glass. Three explanations are suggested to account for the beneficial effects of a feeder layer. First, the bone marrow cells may adhere to the HA cells with greater ease than they do to glass. However, unsuccessful efforts to cultivate bone marrow cells on glass were characterized by an arrest at the round cell stage and not by failure of the cells to attach to glass. Second, autotoxic metabolites formed by the bone marrow cells may be removed by the HA cells. Third, the feeder layer may elaborate metabolites which are beneficial for survival and multiplication of certain cells found in the bone marrow but which either do not accumulate in effective concentration in the medium or are extremely labile at 37°C.

Summary. The use of a feeder layer of x-irradiated or non-irradiated primary human amnion monolayers in cultivation of leukemic and normal human bone marrow aspirates results in establishment of primary, human fibroblastic cell cultures. Comparison of normal and leukemic cultures failed to reveal differences in morphology, type of degeneration, rate of metabolism or viral susceptibility. A cytopathogenic agent was not recovered from leukemic aspirates and use of the fluorescent antibody technic failed to reveal a specific antigen capable of reacting with leukemic sera in leukemic bone marrow cell cultures. Cultures of cells from both leukemic and normal bone marrow supported multiplication of polio, herpes simplex and measles virus. Normal marrow cell cultures supported multiplication of Sindbis and influenza A virus, but not of an egg adapted strain of mumps virus. The reproducible cul-

tivation of cells obtained from human bone marrow provides an accessible source of primary human fibroblastic cell culture that may prove useful in both microbiological and hematological experimentation.

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Occurrence of Antibody in Human Vaginal Mucus.* (26421)

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The occurrence of specific antibody activity outside the tissues proper and often contained in mucous secretions has been described in a number of mammalian species. Pierce has reviewed critically much of the relevant material(1). He refers to such antibody as mucoantibody, implying only that the activity is found to occur admixed with mucoprotein. Such antibody found in saline extracts of feces, and presumably occurring free in the bowel, has been called coproantibody.

The evidence suggests that such antibody in the upper respiratory tract is closely related to, and possibly derived from, circulating antibody. In other instances, notably in the case of antibody found in feces, vaginal secretions, and urine, antibody response following active immunization by the parenteral route appears to differ significantly from serum antibody response in that the activity appears and reaches a peak earlier than serum antibody, and disappears while serum antibody persists. In contrast to serum antibody, such antibody is probably not cumulative, and the observed titers may reflect significantly the rate of antibody formation. Quantitative studies have supported this view(1,2,3), and it is inferred that the antibody is formed locally.

The occurrence of muco-antibody in areas subject to an essentially local infection without invasion of the deeper tissues provides a rational basis for an effective antimicrobial prophylactic immunity, and may also be a factor in the normal microbial flora of such areas. In the first instance, immunity to experimental enteric cholera in the guinea pig (4) and rabbit(5), and similar Shigella infections in the guinea pig(6) and mouse(7) appears to be a function of the presence of antibody in the bowel. Similarly, immunity to Trichomonas infection of the bovine vagina is also associated with presence of antibody in the vaginal mucus(1). In the second, the occurrence of coproagglutinins to coliforms and enteric pathogens in man(8,9, 10) as well as in experimental animals is suggestive in connection with the observed succession of immunologic types of coliform bacilli in man(11).

In addition to the immune response demonstrable in bovine vaginal mucus to Trichomonas infection, antibody to homologous sperm has been found in the rabbit following

* This investigation was aided by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund, University of Chicago.

local application of sperm or testis(12), and in the serum and uterine secretions of both rat and rabbit after introduction of homologous and heterologous sperm into the vagina (13). In man the occurrence of anti-sperm agglutinins in the cervical secretions of human females shortly after ovulation and in the second trimester of pregnancy has been reported(14), and hemagglutinins to Group A and Group B red blood cells has been found in the cervical secretions of Group O women(15).

The present report is concerned with the immune response of the human female to conventional typhoid bacillus vaccine given parenterally, and to soluble typhoid bacillus antigen applied locally, demonstrable as agglutinin in serum and vaginal mucus.

Methods. The immunizing agents were commercial typhoid-paratyphoid (TAB) vaccine, and soluble typhoid bacillus antigen. The latter was prepared by sonic lysis (9 KC/sec for 2 hours) of a heat-inactivated (58-60°C for 30 minutes) saline suspension of an 18 hour culture of *Salmonella typhosa* 901 on veal infusion agar. The lysate was cleared by centrifugation at 4500 × G and the supernatant diluted in saline to contain approximately the same amount of protein nitrogen (Folin-Ciocalteau method) as commercial typhoid vaccine, *i.e.*, 50-60 μg/ml. The agglutinating antigen was a saline suspension of living strain 901; *i.e.*, antibody to both H and O antigens was titrated.

Human female volunteers were immunized by subcutaneous inoculation of a single dose of 0.5 ml of typhoid vaccine in the case of the parenterally immunized group, and the second inoculation was given when vaginal mucus agglutinin was no longer detectable even though serum agglutinin persisted. In the immunization of the group receiving antigen locally, a junior-sized Tampax was soaked with 10 ml of the soluble antigen solution, inserted in the vagina, and allowed to remain for 3 days.

Vaginal mucus was collected by swabbing the cervix uteri and posterior vaginal fornix with a small cotton pledglet, slightly moistened with saline. The pledglet was then washed out in 5 ml of saline, the solution clarified by centrifugation at 3000 × G, and titrated for agglutinin. It was assumed that this supernatant represented a 1:5 dilution of vaginal mucus. Mucus and blood specimens were taken prior to immunization and at weekly or biweekly intervals thereafter, but mucus specimens were not taken during the menstrual period. Postimmunization time was measured as days after first contact with the antigen.

Results. The observed mucus and serum antibody response following primary parenteral inoculation with typhoid vaccine is shown in Table I. It is evident from these data that the titer demonstrable in vaginal mucus was closely similar to that of copro-antibody in its relation to serum agglutinin. In spite of unavoidable irregularity in time of taking specimens, agglutinin was demonstrable in the vaginal mucus of 2 of the 5 subjects by the 3rd day and before serum agglutinin could be detected. The muco-antibody titer reached a peak of 1:100 in all subjects 9-20 days after inoculation, remained at approximately this level until the 4th week, and declined thereafter. It had completely disappeared in 3 of the 5 subjects after 7 weeks, persisted for another week in one subject, and to nearly 4 months in another. This last subject, however, proved to have an hypertrophic endometrium with constant slight oozing of blood, and the muco-antibody status of the observed agglutinin is questionable. Serum agglutinin persisted in all subjects over the entire period of observation.

In the secondary response, elicited by a second parenteral inoculation of vaccine after complete disappearance of agglutinin from vaginal mucus, the vaginal mucus agglutinin again appeared as early as the 4th day in 2 of the subjects. While peak titer was first reached in mucus and serum at 20 and 22 days postimmunization on the average in the primary response, in the secondary response peak titers were reached at 23 and 41 days respectively to give a marked discrepancy between vaginal mucus and serum response. Although vaginal mucus agglutinin titers were somewhat higher than in

TABLE I. Agglutinin Response to Parenteral Typhoid Vaccine.

Immune response		Days after inoc.	Subject A (1940)* Mucus	A (1940)* Serum	B (1957)* Mucus	B (1957)* Serum	C (none)* Mucus	C (none)* Serum	D (none)* Mucus	D (none)* Serum	E (none)* Mucus	E (none)* Serum
		0	0	0	0	0	0	0	0	0	0	0
		3	10		10		0		0		10	
		7			10	0	10	10			10	0
		10			50		100				50	
	Primary	17	50	200	100	200	50	1000	100	2000		
		21	100						100			
		24	100	5000			100	500	100	2000	100	500
		28			100		50		100		100	
		31	10	500	10	200	20	500				
		33									100	500
		35	10		10		50				50	
		51	0		0		0		50	1000	50	
		0	0	200	0	500	0	500	0	20	0	
		3	0		0		20		200			
		7	0	200			20	1000	200	1000	100	500
		10	20				20		100			
		14	50	200	20	100	100	500			100	
		17	50		100						200	
		21			100	200					200	
		24			100		100		10	1000	200	
		28					50					
	Secondary	31	50		50		50		10		200	1000
		35	100	500			200		50	500		
		38	10		50		20		20			
		42			100	2000	50	1000			200	
		45	0									
		47									100	
		49	50		10	200			0			
		51									100	5000
		52	20	1000	0		10					
		54									20	
		56			0	100						
		63	0	1000			100	500				
		68									0	
		70					0					
		72									0	5000

* Date of prior typhoid immunization.

the primary response, to as much as 1:200 in 3 cases, they did not persist longer, and, as in the primary response, disappeared while serum titers persisted at levels of 1:100 to 1:5000 (Table I). Of the subjects, 2 had had earlier conventional typhoid immunization, 2 had not, so far as could be determined, and in one case it was not known whether or not there had been prior immunization.

The immune response of a group of 8 subjects to locally applied soluble typhoid antigen appeared to differ significantly from that to parenteral vaccine. Relatively high agglutinin titers, 1:100 to 1:200, were found in 3 subjects in the first mucus specimens taken and at the end of the 3-day period of exposure to the antigen. Peak titers were reached in an average of 15 days and were very high with 7 of the 8 subjects showing an agglutinin titer of 1:2000. In general, serum agglutinin response was inferior to that obtained with parenteral vaccine, except in 2 subjects who had a history of recent vaccination (Table II).

Discussion. In the 2 earlier reports of the presence of antibody in human vaginal mucus noted above, *viz.,* sperm agglutinins and blood group antibodies, the status of the antigenic stimulus is somewhat uncertain in the former and presumably nonexistent in the latter. The study reported here is concerned with a model system in which the antigen is a conventional immunizing preparation, and the microorganism does not nor-

TABLE II. Agglutinin Response to Locally Applied Soluble Antigen.
(Immune response)

Days after inoc.	Subject															
	Gl (1952)*		Hi (1955)*		St (none)*		We (1954)*		Co (none)*		Com (1953)*		Mo (1960)*		Bl (1960)*	
	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S
0	0	0	0	0	0	0	0	0	0	0	0	0	0	500	0	5000
3									100		200				200	
4	0		0		200		2000	5000								
6									200	200	500	2000				
7					100	200	1000									
8	100		200	200												
11	100	500			1000		1000						500	2000		
13			100						500	100	100	2000			1000	
14					100	200	200	500								
15	2000												2000			
17									2000		2000					
18	500	100			2000		500						200	1000		
20									20	20	0	500				
21					0	0										
22	1000		2000	200									1000			
24							20	1000	20		0					
25	20		0										20	1000		
27					100	100			200	1000	50	500			0	500
28	20		0	1000			20						20			
32	200	200	50												50	
43	1000														20	

* Date of prior typhoid immunization.

M = mucus; S = serum.

mally occur in the vagina. The observed results would seem, therefore, to establish the occurrence of specific antibody in human vaginal mucus as a part of the active immune response.

While it is probably not possible to differentiate a primary and secondary response because of the frequency of typhoid immunization and intercurrent infections with Salmonella types having antigens in common with the typhoid bacillus, the evidence presented here suggests that primary and secondary responses do not differ greatly with respect to vaginal mucus agglutinin although they do in serum antibody response. It was of interest too that neither the numerous menstrual periods, nor the 2 pregnancies which intervened in the soluble antigen group, nor the single pregnancy in the secondary response group, had any apparent effect on the rise and fall of antibody titer in the vaginal mucus.

The similarities in behavior of the titer of vaginal antibody in relation to serum antibody to that of coproantibody are striking. Its relatively rapid appearance and disappearance and seeming independence of serum antibody would suggest that here also the nonaccumulative titer may be a measure of rate of antibody formation, and that the observed antibody is formed locally. The case for local formation of vaginal antibody is strongly supported by the superiority of this immune response to locally applied antigen, and the relatively poor serum antibody response to this kind of antigenic stimulus.

Whether or not antibody present in vaginal mucus is a factor in the composition of the normal microbial flora of the vagina, is a significant element in effective immunity to vaginal infections, or even may relate to the viability of sperm, is not clear.

Summary and conclusions. Using typhoid vaccine, soluble typhoid bacillus antigen, and specific agglutinin as a model antigen-antibody system, it has been possible to demonstrate the occurrence of specific antibody in human vaginal mucus as a consequence of active immunization. Vaginal antibody was produced in response to either parenteral or locally applied antigen and the response to the latter kind of antigenic stimulus was superior to that of the former. Vaginal agglutinin appeared earlier than serum antibody, reached a peak slightly sooner, and disappeared in 7-8 weeks though serum antibody persisted, often in relatively high titer. Vaginal antibody response to primary and secondary inoculation appeared to be substantially the same, and was not affected by menstruation or intervening pregnancy.

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Systemic Lupus Erythematosus and Normal Antibodies. (26422)

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Systemic lupus erythematosus (SLE) is a disease marked by an abnormality in immunological reactivity and is generally considered to represent an autoimmune disease *par excellence*. The sera of patients with this condition are capable of giving typical serological reactions with cell nuclei, nucleoprotein and deoxyribonucleic acid, cytoplasmic constituents, saline extracts of kidney, liver, and thymus tissues, thrombocytes and clotting factors(1,2). Syphilitic reagin or Wassermann antibody, unrelated to leutic infection, directed against lipid substances of tissue origin is also present occasionally. The serum substances responsible for these reactions are globulins, relatively heat stable and capable of complement fixation; presumably, they are antibodies.

The impression has arisen, therefore, that the SLE patient is a prolific antibody producer. Antibodies described in SLE patients are all directed against autologous antigenic substances, but little evidence is available if enhanced amounts of circulating antibodies to substances antigenically unrelated to mammalian tissue are also present. Recently, 4 of 12 SLE patients have been reported to possess hemagglutinating antibody to penicillin in contrast to the absence of detectable levels of this antibody in 31 other patients after completing a course of penicillin therapy(3). On the other hand, after inoculation with vaccines of brucellae and rickettsiae, antibacterial and antirickettsial antibodies appeared in patients with SLE at comparable times and in comparable titers to those observed in a control group of patients (4).

The present study was undertaken to determine whether the enhanced antibody levels in patients with systemic lupus erythematosus were restricted to autoantigens or whether their altered immunological reactivity extends to other antigenic materials as reflected by so-called normal antibody concentrations. The origin of the blood group isoantibodies and of other normal antibodies against microbes or microbial products is not known. In some instances, they may arise by immunization against the homologous or related microbes which find their way into the tissues during life(5). Other normal antibodies are possibly merely normal physiological constituents of serum which fortuitously possess a complementary configuration to microbial antigens(6). In any event, determinations were made of the relative levels of blood group isoantibodies and of other so-called normal antibodies to a gram-negative bacterium selected at random, *Proteus* OX-2, and to streptolysin O, an enzymic product of the gram-positive *Streptococcus* in a group of SLE patients, other hospital patients and normal individuals.

Materials and methods. Sera. The test specimens consisted of 11 sera from SLE patients all of which showed marked complement fixation reactions with calf thymus nucleohistone antigen(7). Control specimens consisted of 12 sera from hospital patients selected at random in whom clinical and serological evidence of systemic lupus erythematosus was lacking and 12 sera from healthy young men who had passed a rigorous physical examination for entrance into the U. S. Military Academy at West Point, N. Y.

Saline isoagglutinins: Serial dilutions of the serum in 2-fold steps were made in 0.85% NaCl starting at 1/4. Each dilution was contained in 0.1 ml. To each dilution, 0.1 ml of a 2% saline suspension of group A_1 red cells was added and the test tubes containing the mixtures were shaken and allowed to stand at room temperature for 30 min, centrifuged lightly (500 g) for 2 min and read macroscopically with the aid of a microscope mirror. The reciprocal of the highest dilution

TABLE I. Blood Group Isoagglutinin Titers in SLE Patients, Other Patients and Normal Individuals.

Anti-A

SLE			Other patients			Normal		
Serum No.	Blood group	Saline titer	Serum No.	Blood group	Saline titer	Serum No.	Blood group	Saline titer
1	O	32	3	B	64	3	B	64
5	O	256	4	O	128	4	B	32
10	O	32	6	O	32	5	O	64
11	B	8	8	O	16	6	O	32

Anti-B

SLE				Other patients			Normal			
Serum No.	Blood group	Saline titer	Indirect Coombs	Serum No.	Blood group	Saline titer	Serum No.	Blood group	Saline titer	Indirect Coombs
1	O	16		1	A	8	1	A	64	
2	A	32		2	A	64	2	A	16	
3	A	8	32	4	O	32	5	O	8	32
4	A	8	16	5	A	8	6	O	2	8
5	O	8	8	6	O	32	7	O	32	64
6	A	16	16	7	A	8	8	O	16	32
8	A	128		8	O	16	9	O	32	
10	O	64		9	O	16	11	A	8	
11	B	32		10	A	8	12	O	8	
				12	O	16				
Geometric mean		22				16			14	

Note: SLE sera No. 7 and 9 and normal serum No. 10 were from Group AB patients. Other patients serum No. 11 was from a Group B individual.

giving visible agglutination was considered the titer. A limited number of sera were also subjected to the indirect Coombs technic. The unagglutinated saline suspended cells were washed 3 times, and one drop of anti-human serum was added to each tube. The tubes were then allowed to incubate at 37°C in a water bath for 30 min, and final readings were made after centrifugation in the same manner as for the saline isoagglutinins. *Antistreptolysin O* determinations were performed with streptolysin O reagent, product of Difco Laboratories, as described by Rantz and Randall(8). *Proteus* OX-2 agglutinin levels were determined by mixing equal volumes of 2-fold serial dilutions of test sera with the bacterial suspension and incubating the mixtures at 52°C for 16 hours. The tests were read macroscopically and the titers determined in a standardized manner (9).

Results. Blood group isoantibodies. Anti-A isoagglutinin determinations were made on sera from individuals of blood groups O and B and anti-B determinations on groups O and A. This was necessary because of the absence of detectable anti-A in the blood groups A and AB individuals and absence of anti-B in those of blood groups B and AB. The results of the isoagglutinin determinations are given in Table I. The differences between SLE and other sera were obviously not marked. Mean anti-A level of the SLE sera was not significantly lower than those of the other groups and although anti-B levels were higher with the SLE sera, analysis of variance of anti-B results indicated greater variation within the 3 groups of sera than between them. Moreover, the indirect Coombs test results performed on 4 random SLE and 4 normal sera (Table I) did not indicate that SLE patients produced inordinately large amounts of incomplete antibody.

Proteus and Streptolysin O antibody determinations. The results of the tests for *Proteus* OX-2 agglutinins and antistreptolysin O (Table II and III) demonstrated that SLE patients do not possess elevated levels of these antibodies; in fact, their antistreptolysin O titers were lower than those of the normal subjects.

Finally, no association was observed be-

TABLE II. *Proteus* OX-2 Agglutinin Titers in SLE Patients, Other Patients, and Normal Individuals.

SLE		Other patients		Normal	
Serum No.	Titer	Serum No.	Titer	Serum No.	Titer
1	<10	1	20	1	20
2	80	2	40	2	20
3	20	3	20	3	10
4	160	4	320	4	80
5	10	5	80	5	10
6	<10	6	20	6	10
7	20	7	20	7	80
8	20	8	20	8	40
9	10	9	<10	9	10
10	40	10	<10	10	20
11	10	11	20	11	<10
		12	20	12	20
Geom. mean*	19		24		19

* Geometric mean.

tween antibody levels determined in this study and nucleohistone antibody levels of the SLE patients. SLE patients No. 2, 3, 11 had nucleohistone complement fixation titers of 243 or greater, whereas serum titers of the other SLE patients were not greater than 27. Yet sera of patients No. 2, 3, 11 were among the lowest in *Proteus* OX-2 agglutinins and antistreptolysin O.

Discussion. SLE has been considered to be a disease associated with an unusually productive antibody synthesizing system. That the SLE patient possesses serum factors, often at extremely high levels, capable of reacting with autogeneous materials has been amply documented. Limited evidence for hyperreactivity, particularly to rare blood group substances(10) and antibiotics (3,11) has also been reported. Although the sample tested was small and the technics have a large inherent error, the results of this study do not, however, lend support to the concept that SLE patients produce abnormally large amounts of circulating antibodies directed against foreign antigens. If the reasonable assumption is made that the exposure of SLE patients to *Proteus, Streptococcus* and blood group antigens was comparable to that of the control groups, then SLE patients do not possess unusual reactivity against these foreign antigens, at least as far as circulating antibodies are concerned. The studies involving deliberate immunization with bacterial and rickettsial vaccines(4) provide even stronger evidence for this premise. However, if normal antibodies determined in this study are unrelated to immune processes, the conclusion must be reached that the cells or processes involved in their production are probably normal in the SLE patient in contrast to other immunological abnormalities in these patients. It would seem reasonable, therefore, to investigate the possibility that the most significant immunological aberration in SLE patients will be found in the delayed or cellular hypersensitivity reaction or in reactions unknown at present. Preliminary findings for an autoimmune reaction of a delayed type to leucocyte homogenates in SLE patients has been reported(12), but it would be desirable to extend these observations to foreign antigens.

TABLE III. Anti-Streptolysin O Titers in SLE Patients, Other Patients, and Normal Individuals.

SLE		Other patients		Normal	
Serum No.	Titer	Serum No.	Titer	Serum No.	Titer
1	12	1	166	1	12
2	12	2	100	2	12
3	12	3	250	3	125
4	100	4	125	5	12
5	100	7	125	7	12
6	166	8	100	9	50
7	12	9	12	10	12
8	<12	10	333	11	100
9	12	11	166	12	100
11	<12	12	125		
Geom. mean*	21		118		29

* Geometric mean.

The sera not tested were in insufficient quantity for the anti-streptolysin O determination.

These results that indicate that SLE patients do not produce abnormally large amounts of circulating antibody to foreign antigens may be related to certain phenomena in immune tolerance. Immune tolerance is relatively easily induced to implantable cells and to soluble antigens of vertebrate origin but impossible with most microbial antigens(13). Thus, normal individuals do not ordinarily form autoantibodies because they are "exposed" to their own cells *in utero* and thus become incapable of reaction against these cellular antigens. In SLE

patients, certain immunologically competent cells may "forget" their early exposure by mutation to a pattern corresponding to a tissue antigen with a concomitant loss of homeostatic controls on number of cells(13). Precisely how such cells arise and proof of their existence remain to be determined. On the other hand, the abnormal immunological reactions in SLE may result simply from autologous antigens altered by contact with microbial agents or their toxins. Such alterations may change certain cellular structures so that they are no longer recognizable by antibody producing cells, and these altered autologous antigens may provide the direct incitant for autoimmune disease. The antibodies produced may be cross reactive with normal structures and thus the auto-immunization process initiated. Certain disease processes elicit elevated levels of tissue autoantibodies(14), but whether these autoantibodies are similar to those seen in SLE patients remains to be determined. The normal agglutinin and antistreptolysin O levels found in SLE patients in this study represent antibodies to foreign antigens and cannot contribute to the question whether the etiology of autoimmune disease is to be attributed to altered normal cellular constituents. The results indicate, however, that SLE patients are not greatly abnormal in their production of so-called normal antibodies to foreign antigens.

Summary. SLE patients do not possess higher levels of blood group isoagglutinins, *Proteus* OX-2 agglutinins or antistreptolysin O than other patients or normal individuals. The immunological hyperreactivity of these patients may be restricted, therefore, to circulating autoantibodies and possibly to the delayed or cellular hypersensitivity reaction.

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Localization of Acetylcholinesterase in the Neurohypophysis and its Functional Implications.* (26423)

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The neurosecretory theory of functioning of the hypothalamico-neurohypophyseal system is now generally accepted. According to this concept, vasopressin and oxytocin, or their precursors, are elaborated within cell bodies of neurons of the supraoptic and paraventricular nuclei, and transported *via* the

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corresponding axons through the infundibular stalk to axonal swellings (the Herring bodies) and terminations in the neurohypophysis, where hormones are stored until secreted(1). Secretion of individual hormones in response to physiological and artificial stimulation is mediated by neural pathways which converge in the hypothalamus. On the basis of pharmacological evidence, it is likely that cholinergic neurons are involved in bringing about secretion of both vasopressin(2,3) and oxytocin(4). Since known cholinergic neurons stain selectively for acetylcholinesterase (AChE) by the acetylthiocholine (AThCh) method(5,6), Abrahams *et al.* (7) examined serial sections of several regions of the dog hypothalamus stained by this technic in order to identify cholinergic pathways in question. Unexpectedly, perikarya of supraoptic and paraventricular neurons themselves were found to be stained distinctly for AChE, but fibers terminating upon them showed no significant staining; among other possible interpretations, it was suggested that the same neurons might liberate at the neurohypophyseal level both acetylcholine (ACh) and endocrine products, with the former providing the stimulus for release of the latter. Recently the same proposal was offered independently by Gerschenfeld *et al.*(8) to explain their electron microscopic observations that individual neurosecretory axonal terminals in the toad neurohypophysis contain 2 distinct populations of granules: (a) large, electron-opaque granules, which could be traced back through the infundibular stalk to the hypothalamus, and presumably represented endocrine secretions, and (b) small, less dense vesicles, confined to terminals, which resembled synaptic vesicles noted previously at various presynaptic terminals where they may represent packets of neurohumoral transmitters(9), including ACh(10).

Since the neurohypophysis itself had not been examined in the histochemical study of AChE of the hypothalamic nuclei(7), it was of considerable interest to determine whether tracts and terminals of the hypothalamico-neurohypophyseal fibers contain significant concentrations of the enzyme.

Materials and methods. The neurohypophysis and infundibular stalk from 7 cats were stained selectively for AChE and nonspecific cholinesterase (ChE) or butyrocholinesterase (BuChE) activities; in most cases, intermediate lobe and adenohypophysis and part of the adjacent hypothalamus were left attached.† The hypophysis was usually sectioned immediately after sacrifice of the cat and excision; in a few instances, it was frozen in isotonic saline solution and used the following day. Fresh frozen sections were cut in the sagittal plane generally at 15 μ (occasionally at 20 μ) thickness, placed on slides, and carried through the standard staining procedure(5,6). In one instance, all 10 combinations of substrates and inhibitors were employed to determine the possible presence in tissues under study of non-cholinesterase enzymes which can attack thiocholine esters used as substrates(6); however, none was detected. Except for the first exploratory specimen, where no inhibitors were used, other specimens were stained with various inhibitor-substrate combinations which always included pre-incubation with di*iso*propylfluorophosphate (DFP) and incubation with AThCh for the selective localization of AChE. Incubation times were varied from 30 to 240 minutes. Following development with $(NH_4)_2S$, which converts the white precipitate of copper thiocholine sulfate at sites of enzymatic activity to copper sulfide, and gold-toning, sections were dehydrated and mounted in Permount. Occasional sections were counterstained with hematoxylin-eosin.

Neurohypophyses from 3 cats were fixed in Bouin's fixative, frozen-sectioned, and stained for neurosecretory substance (NSS) by Bargmann's modification of Gomori's chrome-hematoxylin method, as given by Pearse(11).

Results. Findings were quite consistent in all 7 hypophyses studied. The distribution of AChE in the neurohypophysis was

† We wish to thank Drs. S. D. Erulkar and E. Koenig for help in obtaining the specimens. Photomicrographs were taken by Mr. E. F. Glifort.

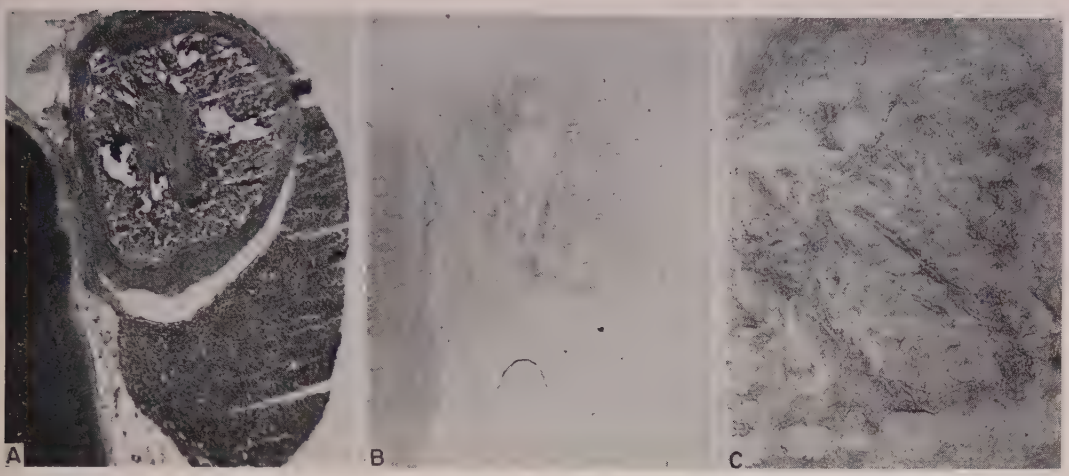

FIG. 1. Section (15 μ) of cat neurohypophysis (above), adenohypophysis (below), and adjacent hypothalamus (left) stained for AChE activity by 120 min. incubation in AThCh medium preceded by 30 min. incubation with 10^{-8}M DFP for selective inhibition of non-specific ChE. A. Magnification $\times$ 13, counterstained with H & E. B. Magnification $\times$ 13, no counterstain. C. Portion of neurohypophysis, magnification $\times$ 80, no counterstain. All staining in B and C represents AChE activity.

observed most precisely in 15 μ sections stained by incubation with AThCh for 120 minutes following pre-incubation with 10^{-8} M DFP for 30 minutes. Such a preparation, counterstained with hematoxylin-eosin, is shown in Fig. 1A, and without counterstaining in Fig. 1B; in the latter, and in the corresponding photomicrograph at higher magnification (Fig. 1C), all staining represents AChE activity.

Fiber tracts of the infundibular stalk showed very faint staining. Throughout the infundibular process of the neurohypophysis, there was light but definite staining (Fig. 1B), which appeared to represent fiber tracts (Fig. 1C) along which were seen numerous vesicular swellings reminiscent of Herring bodies (12, plate VI) seen with specific staining for NSS. For comparison, sections of the superior cervical ganglion and nodose ganglion were stained simultaneously under identical conditions. Neurohypophyseal fibers were stained with an intensity similar to that of the afferent vagal fibers of the nodose ganglion; in both these types of fibers, intensity of staining for AChE was considerably lighter than that of the cholinergic preganglionic fibers, but much greater than that of the virtually unstained postganglionic adrenergic fibers of the superior cervical ganglion; photomicrographs of these structures stained identically, have been published previously(6).

Varying degrees of staining for AChE were noted in adjacent hypothalamic tracts and nuclei, and intense staining was present in scattered nerve trunks in the immediate vicinity of the hypophysis. However, intermediate and anterior lobes of the pituitary appeared to be virtually devoid of AChE activity (*cf.* Figs. 1A and 1B). The only distinct staining for non-specific ChE (BuChE) noted in the area of the neurohypophysis was in rather poorly defined structures at its borders; these have been identified tentatively as interstitial cells. Similarly stained structures were noted occasionally in and at the periphery of the adenohypophysis.

The pattern of deep purple staining obtained with the chrome-hematoxylin method for NSS was similar to that for AChE, and, while less distinct, resembled that seen in published photomicrographs (12, plate VII).

Discussion. The present results, along with those of an earlier study in which AChE was demonstrated histochemically in neuronal cell bodies of paraventricular and supraoptic nuclei of the dog(7), indicate that enzyme is present in moderate concentrations throughout the lengths of the neurons com-

prising the hypothalamico-neurohypophyseal tract. In intensity of staining, they resemble the afferent vagal neurons, and contrast with both the known cholinergic neurons of the cat, which contain uniformly high concentrations of AChE, and with the majority of adrenergic neurons of the same species, which contain practically none. Although it is generally considered that the neurohumoral transmitter of primary afferent fibers is not ACh, recent work has suggested that in response to stimulation an ACh-like compound was liberated by the central terminals of vagal afferent fibers in the superior cervical ganglion of cats in which functional cross-anastomosis had been accomplished with the peripheral preganglionic cervical sympathetic trunk.‡ In connection with the latter findings it was suggested that under normal conditions, ACh might represent not the primary neurohumoral transmitter of vagal afferent fibers, but that its release and action immediately at presynaptic terminals might promote release of an unidentified synaptic transmitter. The present findings are consistent with previous proposals(7,8) of operation of a similar mechanism at terminals of the hypothalamico-neurohypophyseal tracts, *i.e.*, that in response to impulses conducted along the axons from hypothalamic nuclei, ACh is liberated at terminals within the neurohypophysis where it brings about the release of oxytocin or vasopressin, and that this local action of ACh is terminated by its hydrolysis by AChE of the axonal terminals. In this situation there is, of course, no postsynaptic site at which ACh could act. The function of perikaryonal AChE in the neurons under discussion, and in acknowledged cholinergic neurons, remains conjectural. It was proposed earlier(13) that it might represent recently synthesized enzyme in transit to its functional site at axonal terminals, *i.e.*, a sequence similar to that of neurosecretory processes as described above. However, recent study(14) has failed to confirm this suggestion.

Summary. Neurohypophyses of 7 cats were stained for AChE activity by AThCh method. In the infundibular stalk, fibers showed light staining, whereas fibers, their vesicular swellings, and terminations showed moderate staining in the infundibular process of the posterior lobe. No significant AChE activity was noted in the anterior or intermediate lobe. Thus, in conjunction with findings of an earlier study(7), neurons of the hypothalamico-neurohypophyseal tract appear to contain light to moderate concentrations of AChE throughout their full lengths. These findings are consistent with previous proposals(7,8) that liberation of endocrine secretions of the neurohypophysis is controlled by liberation of ACh from the same terminals.

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Blood Group Substances in Canine Gastric Mucus and Acid Secretion.*† (26424)

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Gastric secretion is considered a major source of blood group substances(1); secretions from cow, horse, pig and man have yielded considerable information in this connection. Investigations into the chemistry of the macromolecular constituents of gastric secretions from dogs(2,3) and of dogs' erythrocyte-isoantibody reactions(4) have not yielded information about canine blood group substances. Because of the availability of methods for collecting and fractionating a variety of uncontaminated canine gastric secretions(5), and for studying such materials in canine as well as in human erythrocyte-isoantibody systems, the present investigation of reactions of canine gastric secretions in both these immunological systems was undertaken.

Methods. Canine gastric secretions. An acid gastric mucus was obtained from mongrel Heidenhain stomach pouch dogs, following topical application of acetylcholine. Acid gastric juice following stimulation by histamine and mecholyl together was obtained by drainage from pouches, and by oro-gastric intubation from dogs with unoperated stomachs.

Enrichment for mucopolysaccharides. The acetylcholine mucus was processed by peptic digestion, followed by phenol extraction of the dialyzed digest. The criterion for mucopolysaccharide enrichment was a lowering of the N:hexosamine molar ratio. Endogenous pepsinogen contained in the mucus was activated by adjustment to pH 2 with dilute HCl, and the mixture was incubated under toluene at 37°C for one week; following this, it was dialyzed, reacidified and incubated for an additional week. This treatment solubilized almost all of the insoluble gel, and after dialysis and alcohol precipitation afforded a moderate degree of mucopolysaccharide enrichment (about 3-fold). From 3-10% of the organic substances initially present in the mucus was insoluble in water and in sodium acetate at pH 8 after peptic digestion; the insoluble debris exhibited molar ratios of 6.8 for N:hexosamine and 4.2 for N:reducing power, but it was set aside and not worked up further because its insolubility rendered it unsuitable for hemagglutination-inhibition studies. The soluble fraction was lyophilized, then subjected to phenol extraction(6). The phenol-soluble (PS)‡ fraction was subjected to ethanol precipitation, using concentrations up to 25% alcohol in presence of sodium acetate. The phenol-insoluble (PI) residue and the PS alcohol precipitates, were washed with alcohol and ether prior to assay.

Chemical assays. Nitrogen was determined by a micro-Kjeldahl procedure(7). Hexosamine was determined by the method of Blix(8) after hydrolysis in 2N H_2SO_4 for 4 hours in a boiling water bath. Reducing power(9) also was determined on the hydrolysate. Hexuronic acid and fucose determinations were made on the unhydrolyzed enriched mucopolysaccharide according to the methods of Dische(10,11). Paper electrophoresis of the enriched products was performed with the Spinco vertical electrophoresis apparatus in borate buffer $\Gamma/2 = 0.1$, pH 9.0, 200 volts, 6½ hours. Strips were stained by PAS(12) and by toluidine blue(13).

Canine immunological systems. Antisera:

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‡ Abbreviations: N = nitrogen; PI = phenol-insoluble; PS = phenol-soluble.

Isoantibodies for the dog blood group antigens A, C, and D were prepared and employed as described by Young *et al.*(4,14). Antisera for hemagglutination inhibition studies were employed both undiluted and at appropriate dilutions with isotonic saline. *Test red cells*: Three per cent suspensions of red cells in isotonic saline were used for study of the canine C and D systems; red cells suspended in saline or in fresh autogenous serum were used for studies of the canine A system. *Test substances*: Dialyzed and lyophilized gastric secretions and fractions derived therefrom were hydrated at levels of 2-10 mg/ml in phosphate buffer (pH 7.4) for 2 hours at room temperature prior to testing. *Agglutination inhibition studies*: Agglutination inhibition tests were performed by preparing dilutions of both antibody-containing sera and the hydrated test substances; these were combined in a "block titration pattern" (15). Test samples were assayed at 1:1, 1:10, 1:100 and 1:1000 dilutions. Canine anti-A serum was employed undiluted and at 1:5, 1:15 and 1:45 dilutions. In all systems, tubes containing antiserum and test substance were incubated for 15 minutes at 37°C, 15 minutes at room temperature and 30 minutes at 4°C in succession, and finally returned to room temperature. Then 0.1 ml of the test red cell suspension was added to each tube. The tubes with suspensions in saline were incubated at room temperature, those with suspensions in fresh autogenous serum at 37°C. Indirect antiglobulin tests were performed on all tubes of the A-system tests which did not show agglutination. Canine anti-C and anti-D sera were used undiluted and at 1:4, 1:12 and 1:36 dilutions in saline, and these test systems were incubated at room temperature after addition of red cells suspended in saline. Antiglobulin tests were not employed in studies of the canine C- and D-systems, because these antibodies do not react with the antiglobulin reagents.

Human immunological systems. Antisera: Group O, Rh negative serum diluted 1:4 with isotonic saline was used for tests of the human anti-A system. Serum of a person of "Bombay type" was used undiluted for anti-H studies. Tests for H substance were performed also with Ulex europeus diluted 1:4 with saline (courtesy of New York City Dept. of Health). *Test cells*: Three per cent suspensions of group A_1 red cells in saline were used for studies of the human anti-A system, and 3% suspensions of group O red cells in saline for studies with anti-H. *Test substances*: Gastric secretions and fractions were tested at only one concentration. Indirect Coombs tests were not performed. These substances and saline controls were incubated with human blood group antisera at room temperature for one hour, then 0.1 ml of the appropriate cell suspension was added. Following this, tubes containing complete test systems were incubated at room temperature for one hour and examined.

Results. The data (Table I) show that gastric secretion from 11 mongrel dogs did not exhibit blood group substance activity in any of the canine test systems. Peptic digests of gastric secretion, PI residues, and ethanol-precipitated preparations of the PS fraction were all without inhibitory activity at levels of 2-10 mg/ml. By contrast, all of the above preparations showed activity in human A and H systems. PI residues and PS-25% ethanol precipitates exhibited activity in the human A-test system at levels of less than 4 γ/ml with a 1:4 anti-A serum + A_1 cells, and at the 4 γ/ml level in the human H-test system with undiluted anti-H serum (titer 1:32) + O cells.

Data on the chemical composition of mucopolysaccharide-enriched fractions are presented in Table II. From 27 to 48 mg of PI residue, and 11 to 17 mg of PS-25% ethanol precipitate, were obtained from 50 ml of acetylcholine mucus. These products were essentially of the neutral mucoid variety; the molar ratios hexosamine : hexuronic acid, determined on pooled specimens because of an insufficient supply of individual specimens, were 8.4 for the PI residue, and 14.2 for the PS-25% ethanol precipitate. The increase in mucopolysaccharide content as a result of fractionation was accompanied by

TABLE I. Examination of Canine Gastric Specimens for Hemagglutination Inhibition.

Dog	Canine blood type	Gastric specimens		Human inhibition systems†		
		Mu*	Acid	Anti-A	Anti-B	Anti-H
326—pouch	A_1	+	+	Ae	N	Ae
381—unop'd	A_1		+	Ae	N	C
390— "	C		+	C	N	C
392— "	C		+	C	N	C
334—pouch	A_1C	+	+	P	N	C
251— "	A_1C	+	+	C	N	Ae
340— "	A_1C	+	+	C	N	C
388—unop'd	A_1C		+	C	N	Ae
363—pouch	A_1CD	+	+	Ae	N	C
379— "	A_2C	+	+	C	N	C
394—unop'd	A_2C		+	C	N	Ae

Dialyzed and lyophilized gastric specimens were used at levels of 6-10 mg/ml.

* Mu = acetylcholine-stimulated anacid mucus.

† Degree of agglutination inhibition: C = complete; Ae = almost complete; P = partial; N = none. All gastric specimens were tested for hemagglutination inhibition with the canine inhibition systems, Anti-A, Anti-C and Anti-D; no agglutination inhibition was found in any of the specimens.

an increase in blood group substance activity; this was demonstrated by a reduction in the minimum quantity of substance necessary to inhibit hemagglutination in the Ulex system from 100 γ for the untreated mucus to 1 γ for the 2 enriched fractions.

Paper electrophoresis in borate buffer of the PI and PS-25% ethanol precipitate revealed one PAS positive zone which migrated a short distance on the anode side. The PI fraction exhibited 2 anodic metachromatic zones when stained with toluidine blue O, the lighter-staining of which coincided with the PAS positive zone; the PS-25% ethanol precipitate exhibited no metachromatic zones with 1 mg of material on the strip. The presence of metachromatic zones in the PI-residue suggests contamination of the blood group substances by small amounts of acid mucopolysaccharide.

Discussion. These results show that canine gastric secretions do not exhibit blood group substance activity when assayed in the available canine test systems. Employment of the block-titration technic favors establishment of mutually optimal concentrations of antibody and soluble blood group substances. Although a relatively small population of dogs was employed in our investigation, the animals studied reflect all of the common blood group combinations of this species.

The purified fractions as well as the untreated secretions from which they were derived exhibited blood group substance activity in test systems for human A and H substance. Lack of reaction with the canine systems notwithstanding, a biological test based on hemagglutination inhibition in human A and H systems may aid in differentiating and characterizing various preparations derived from canine gastric secretion.

Several possibilities other than absence of canine blood group factors may be advanced to explain the lack of activity in the canine test systems. It is possible that the

TABLE II. Chemical Characteristics of Mucopolysaccharide-Enriched Fractions of Acetylcholine Mucus, Following Peptic Digestion.

	Mean molar ratios (stand. dev.)		
	N:Hexosamine	N:Reducing power	Hexosamine:Fucose
Phenol-insoluble fraction (5 specimens)	3.3 (1.2)	2.1 (.4)	4.1 (.6)
Phenol-soluble fraction precipitated with 25% ethanol (5 specimens)	4.8 (1.7)	3.9 (1.3)	3.9 (.7)

small amount of water-insoluble debris excluded in the present work may have possessed this activity. Mild means for solubilizing this fraction would be required to investigate this aspect further. Also, it is conceivable that agglutination of canine erythrocytes by appropriate antisera depends on the presence of a complex antigen composed of protein components, either alone or in combination with mucopolysaccharide(s). If protein components as well as mucopolysaccharide were necessary to complete the antigen, then gastric mucopolysaccharide alone would not be expected to inhibit the hemagglutination reaction. Further investigation will require liberation, fractionation and concentration of canine erythrocyte surface proteins and protein-mucopolysaccharide complexes.

Summary. Canine gastric secretions—both fluid acid and viscous non-acid—and hexosamine-rich fractions derived therefrom, were assayed for blood group substance activity in canine and in human test systems. None of the specimens tested was active in the canine test systems, but all exhibited A and H activity in the human test systems; no human B activity was detected. Hexosamine-rich fractions obtained by peptic digestion and phenol extraction of canine gastric secretion were largely of the neutral mucoid type, and were active in human A and H systems at the microgram level.

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Anticonvulsant Properties of 1-(1-phenylcyclohexyl) piperidine·HCl and Certain Other Drugs. (26425)

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1-(1-phenylcyclohexyl) piperidine · HCl (PCP) was found to be an extremely active agent in prevention of electrically induced tonic extensor seizures in mice(1). In man PCP has been shown to induce interceptive sensory deprivation at low doses and general anesthesia at high dose levels(2,3). This investigation dealt with a comparison of the anticonvulsant properties of PCP, Dilantin, phenobarbital and barbital in convulsions induced by sound, by pentylenetetrazol and by electroshock.

Materials and methods. The experiments on audiogenic seizures were conducted on DBA/2 male mice, 28 to 35 days old, obtained from the Roscoe B. Jackson Memorial Laboratory. The age range between 4 to 5 weeks was found to be the critical period for audiogenic seizure susceptibility in this strain of animals.

The apparatus consisted of a small insulated plywood chamber (40″ x 32″ x 30″) with a hinged door at the front. In it were wired a 4″ electric fan and 2 electric door

bells. A 12″ x 12″ glass window was inserted in the front door for full-view observation. A Petri dish (4¾″), covered with an inverted cylindrical wire basket (4½″ x 4½″) was used to house each mouse during the experiment.

The sound, approximately 90 decibels, was turned on for 90 seconds. During this period the seizure pattern was observed according to the criteria adopted by Frings and Frings(4) in the following sequence: the running phase, clonic spasms, clonic-tonic seizures with extension of limbs and death. Because the clonic spasms were not consistently seen in all animals between the running and the extensor seizure phase, this seizure pattern was not used in evaluation of the anticonvulsant effect of drugs. Under the experimental conditions employed, violent running in the cage occurred within 10 to 30 seconds during sound stimulation in every mouse; this was usually followed by tonic extension of limbs and death. In 50 untreated mice, 10 animals each from 5 groups received from the supply house at different times during the year, 90% of them at least in each group had extensor seizures and succumbed in respiratory failure. Because of the lethal effect of audiogenic stimulation, it was not possible to examine animals audio-susceptibility prior to drug testing. Consequently, 10 DBA/2 mice from each supply of 120 animals were examined for audiogenic seizures as controls; anticonvulsant activities of drugs as determined with the same group of mice were estimated on basis of the control response. And because of the brief age interval during which DBA/2 mice will respond in convulsions to sound, they were used only once in our experiment. Two mice were on observation simultaneously. Four groups of 10 animals each were employed to examine the drug effect at graded dosages.

Ordinary male Webster albino mice, weighing 18-24 g, were used to study the effect of drugs on pentylenetetrazol and electrically induced convulsions. A 0.9% pentylenetetrazol solution was given intramuscularly at 90 mg/kg, this being a submaximal convulsive dose for mice. In the present study, only suppression of the initial clonic seizures was taken as a measure of anti-pentylenetetrazol activity of drugs(5). The supramaximal shock procedure of Toman, Swinyard and Goodman(6) was followed to produce tonic extensor seizures. A current of 20 milliamperes was applied to animal's ears by clips for 0.2 seconds.

PCP, Dilantin (5,5 diphenylhydantoin sodium), phenobarbital (5-phenyl 5 ethyl barbiturate sodium), and barbital (5,5 diethyl barbiturate sodium) were dissolved in water and administered intraperitoneally. Their anticonvulsant activities were examined at time of pre-determined peak effect. Four graded doses of each drug, which would suppress seizures in 10 to 90% of the mice (10 animals per dose) were used to determine the 50% effective dose. ED_{50} was estimated graphically by the probit-log dose procedure of Miller and Tainter(7). Standard errors of a ratio of the means were computed from individual standard errors in percentages(8).

Results. In Table I are given the doses of drugs which would prevent seizures in 50% of the animals (ED_{50}) in respond to sound, pentylenetetrazol or electrical stimuli. Their relative anti-seizure activities, in reference to that of PCP, are expressed in ED_{50} ratios of molecular equivalents. There is also given for each drug a ratio of ED_{50}'s that were required for suppression of running and for prevention of extension of limbs in audiogenic seizures. This, as indicated by a/b in the table, signifies the relative effectiveness of each drug in combating the 2 types of seizure response. In the last column of the table is shown a comparison by ED_{50} ratios for phenobarbital and barbital of their anti-extensor seizure activity in convulsions produced by electroshock to their anti-clonic seizure activity in pentylenetetrazol-induced convulsions.

It may be seen first in audiogenic seizures that PCP and barbital were the most and the least effective drug respectively in suppressing both running and the extensor phase of the convulsion. Whereas Dilantin and phenobarbital were approximately equally capable of protecting animals from the extension of limbs, the former was less effective than the latter in prevention of running ac-

TABLE I. Anticonvulsant Effect of PCP and Other Drugs in Audiogenic, Pentylenetetrazol- and Electrically-Induced Seizures.

Compound	Time after inj., hr	Audiogenic (a) Running ED_{50} ± S.E., mg/kg	Audiogenic (b) T.F.S.* ED_{50} ± S.E., mg/kg	a/b§	Pentylenetetrazol (c) Int. Cl. S.* ED_{50} ± S.E., mg/kg	Electroshock (d) T.F.S. ED_{50} ± S.E., mg/kg	d/c§
PCP	¼	.73 ± .10 (1)†	.45 ± .07 (1)	1.62 ± .34	Ineffective at 5-40	5.20 ± .30 (1)	
Dilantin	1	8.50 ± 1.65 (.09 ± .02)	3.08 ± .36 (.15 ± .03)	2.76 ± .62	*Idem*	12.4 ± .45 (.43 ± .07)	
Phenobarbital	½	4.44 ± .39 (.18 ± .03)	3.80 ± .57 (.13 ± .03)	1.17 ± .21	31.5 ± 1.1	33.4 ± 1.42 (.18 ± .01)	1.06 ± .06
Barbital	1	16.5 ± 1.5 (.06 ± .01)	15.2 ± .2 (.04 ± .01)	1.08 ± .10	34.2 ± 1.5	218.0 ± 6.20‡ (.03 ± .01)	6.37 ± .34

* Int. Cl. S. = Initial clonic seizures; T.F.S. = Tonic extensor seizures.
† In parentheses, relative activity in reference to PCP on basis of mol equivalents.
‡ Loss of righting reflex.
§ a/b, d/c = ED_{50} ratios indicating relative effectiveness of each drug in combating 2 types of seizures.

tivity. As indicated by the ED_{50} ratios for each drug, a larger dose of PCP or Dilantin was required for suppression of running than that for extensor seizures. On the other hand, doses of phenobarbital or barbital taken for suppression of these 2 seizure responses were the same.

In regard to suppression of pentylenetetrazol-induced clonic seizures, phenobarbital and barbital were equally effective. PCP and Dilantin were ineffective at 5 to 40 mg/kg, the latter being a maximal tolerated dose of PCP in mice. In protecting animals from extensor seizures to electroshock, Dilantin is more effective than phenobarbital. Otherwise, the antiextensor seizure effects of these drugs were in the same order of activity as found in audiogenic seizures.

A striking difference in anticonvulsant properties of phenobarbital and barbital is shown by their ED_{50} ratios of electrically-induced extensor seizures to pentylenetetrazol-induced clonic convulsions. While the ED_{50}'s of phenobarbital for suppression of the 2 types of seizures were the same, the ED_{50} of barbital for extensor seizure was 6 times greater than that for clonic seizures.

Discussion. As shown by its anticonvulsant spectrum, PCP and Dilantin seem to possess certain neuropharmacological properties in common. The inability of both drugs to suppress pentylenetetrazol-induced clonic seizures correlates with lack of a hypnotic action in man. This appears to support the assumption that the central depressant action of phenobarbital and barbital responsible for suppression of pentylenetetrazol-induced clonic seizures is the same as that for induction of hypnosis(9). PCP, on the other hand, possesses an anesthetic action which is absent in Dilantin. The fact that PCP will produce sensory deprivation in man at low doses and general anesthesia at high dosages points to the possibility that this drug acts on both the afferent and the efferent nervous system in suppression of audiogenic seizures.

Summary. 1-(1-phenylcyclohexyl) piperidine · HCl was found to be a very effective agent in suppression of audiogenic seizures in mice. It was approximately 7 times more potent than Dilantin and phenobarbital and 25 times more potent than barbital. PCP was likewise the most active anticonvulsant in electrically induced tonic extensor seizures. Like Dilantin, on the other hand, PCP was devoid of any anti-clonic seizure activity in pentylenetetrazol-induced convulsions. A comparison was also made of the anticonvulsant effects of Dilantin, phenobarbital and barbital in audiogenic, pentylenetetrazol and electrically induced convulsions.

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Effect of Protein on Utilization of Vitamin A in the Chick.* (26426)

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Conflicting observations have been reported on the effect of dietary protein level upon vit. A requirement. Baumann *et al.* (1) observed that rat diets low in protein reduced the storage of vit. A in the liver and increased rate at which stored vitamin A was depleted. Arnich and Pederson(2) found the concentration of vit. A per gram of liver to be almost doubled in rats receiving diets low in protein as compared to faster-growing rats receiving adequate protein; the total vit. A per liver, however, being approximately equivalent. Dye *et al.*(3) observed little effect upon utilization of vit. A by rats, regardless of dietary protein level.

On the other hand, Mayer and Krehl(4) observed in rats fed diets deficient in vit. A, that as dietary protein increased, there was a concomitant increase in severity of vit. A deficiency symptoms. Bohman *et al.*(5) observed a reduced level of vit. A and carotene in the liver and blood plasma when extra protein supplement was fed to wintering beef calves. Olsen *et al.*(6), using Columbian Rock progeny of hens maintained on diets low in vit. A, observed that as dietary protein in a practical chick diet increased from 16.9% to 24.6%, liver storage of vit. A in the chicks decreased about 40%.

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In view of the lack of agreement on the effects of dietary protein levels upon vit. A requirements, experiments were undertaken in our laboratory to study this relationship in the chick, and to investigate blood uric acid levels of chicks fed various amounts of protein and vit. A, in an effort to extend the findings of Elvehjem and Neu(7) and Stoewsand and Scott(8) as to factors concerned in producing elevated blood uric acid levels, and to determine if these levels can be used as a criterion of assessment of vit. A deficiency in the chick.

Methods. Male White Plymouth Rock chicks were randomly distributed among the various lots at one-day of age, using duplicate lots of 10 chicks per treatment in Experiment 1 and duplicate lots of 14 chicks per treatment in Exp. 2 and 3. Chicks in Exp. 1 were fed *ad libitum*. At the end of one week, 12 chicks per lot in Exp. 2 and 10 chicks per lot in Exp. 3 were selected according to uniformity of weights within each lot, and continued on treatment for the subsequent 3 weeks. To achieve approximately equivalent growth, the feed consumption of the chicks in Exp. 2 and 3 was limited during this 3 week period by equalized paired feeding by groups to that of the chicks receiving the highest protein treatment within each level of vit. A supplementation.

The diets are presented in Table I. Protein levels were 71.5% (high); 47.5% (mod-

TABLE I. Basal Diets.

Ingredients	Protein levels: High	Moderately high	Moderate	Low
	%			
Cerelose	—	30.23	60.55	71.05
Isolated soybean protein (ADM)	83.94	54.50	25.09	14.59
Cellulose	3.00	3.00	3.00	3.00
Stripped lard	3.00	3.00	3.00	3.00
Mineral mix*	6.13	6.13	6.13	6.13
Vitamin mix†	1.00	1.00	1.00	1.00
DL-Methionine	1.20	1.20	.70	.70
Cystine	1.00	.30	—	—
Glycine	.50	.40	.30	.30
Choline Cl	.22	.22	.22	.22
	μg/kg			
Vitamin B_{12}	50	35	20	20
Santoquin	.0125	.0125	.0125	.0125
Protein, %	71.5	47.5	21.9	12.9
Metabolizable energy/g	3.30	3.30	3.30	3.30

* g/100 g of diet: $CaHPO_4$, 2.15; $CaCO_3$, 1.49; KH_2PO_4, .867; NaCl, .8; $MgSO_4$, .25; $FeSO_4 \cdot 7H_2O$, .0333; $MnSO_4 \cdot H_2O$, .0333; KI, .00026; KCl, .5; $CuSO_4 \cdot 5H_2O$, .00167; $ZnCl_2$, .0114; $CoCl_2 \cdot H_2O$, .00017; $Na_2MoO_4 \cdot H_2O$, .00083.

† mg/kg of diet: biotin, .2; menadione sodium bisulfite, 1.0; pyridoxine, 4.5; folic acid, 4.0; thiamine, 10.0; riboflavin, 10.0; Ca pantothenate, 20.0; niacin, 50.0; inositol, 250.0. Units/kg: vit. D_3, 975; d-α-tocopheryl acetate, 66.

erately high); 21.9% (moderate) and 12.9% (low). Each of these protein levels was fed with graded levels of vit. A as indicated in the individual experiments. Liver vit. A was determined according to the procedure of Ames *et al.*(9); blood uric acid by the method of Brown(10); serum vit. A by the method of Garbers *et al.*(11). Excreta vit. A was determined after eluting the ether extract of the non-saponifiable material through an alumina column according to a method described by Thompson *et al.*(12).

Results and Discussion. Experiment 1. The experimental plan and results are presented in Table II. These results show that the vit. A requirement of the chicks increased as protein content of the diet was increased. This was evidenced by increased mortality and decreased vit. A liver stores in chicks receiving similar amounts of vit. A, but increasing amounts of protein. Results of the blood uric acid analyses are presented in Table III. A high blood uric acid level was observed in the chicks fed the high protein diets regardless of vit. A content of the diet. On the moderate protein diet without added vit. A, 75% of the chicks showed the ataxic symptoms characteristic of vit. A deficiency, yet no increase occurred in the uric acid content of the blood. Thus it appears that blood uric acid in the young chick does not necessarily increase in acute vit. A deficiency.

Experiment 2. The experimental plan and results are presented in Table IV. Since Popper and Greenberg(13) reported that vit. A is bound to certain fatty materials within

TABLE II. Effect of Dietary Protein and Vit. A Level upon Chick Growth and Liver Storage of Vit. A (Exp. 1).

Treatment: Protein* level	Vit. A level, USP units/kg	4 wk wt, g	USP units liver vit. A: Per g	Per liver
High	0	All dead	0	0
Moderate	0	" "	0	0
Low	0	257 (6/20)†	0	0
High	440	235 (4/20)	0	0
Moderate	440	483 (18/20)	0	0
Low	440	308 (20/20)	0	0
High	5280	350 (20/20)	2	23
Moderate	5280	536 (20/20)	35	680
Low	5280	327 (20/20)	51	617

* Percentage protein in each diet is presented in Table I.

† Figures in parentheses show No. of chicks alive at 4 wk of age over No. of chicks at start. Most of the chicks that died showed severe ataxia of vit. A deficiency prior to death.

TABLE III. Blood Uric Acid of Chicks Receiving Various Levels of Dietary Protein and Vit. A.

Treatment: Protein level	Vit. A level, USP units/kg	Age, days	Blood uric acid, mg %
High	0	19	11.7 ± 2.2*(70%)†
Moderate	0	19	4.4 ± 1.0 (75%)
Low	0	19	2.2 ± .4
High	440	24	16.0 ± 1.5 (33%)
Moderate	440	24	5.2 ± .6
Low	440	24	2.4 ± .2
High	5280	29	16.3 ± 1.0
Moderate	5280	29	3.8 ± .6
Low	5280	29	3.1 ± .3

* Stand. error of mean.

† No. of chicks showing symptoms and mortality due to vit. A deficiency.

TABLE IV. Effect of Dietary Protein upon Blood Ascorbic Acid, Liver Lipids and Liver Storage of Vit. A (Exp. 2).

Treatment					
Protein level	Vit. A level, USP units/kg	4 wk wt, g	Total liver vit. A, USP units/liver	Total liver lipids, %	Blood ascorbic acid, mg %
High	440	249 (19/24)*	0	—	1.45
Moderately high	440	289 (23/24)	0	—	1.92
Moderate	440	293 (24/24)	0	—	2.37
High	5280	331 (24/24)	41	4.6	1.67
Moderately high	5280	356 (24/24)	203	4.8	1.49
Moderate	5280	342 (24/24)	431	4.3	2.05

* No. of surviving chicks over No. at start.

the liver, measurements were made of the total liver lipids in the chicks receiving the 3 levels of protein. Furthermore, in view of the report by Mayer and Krehl(4) that vit. A deficiency in the rat decrease the blood, adrenal and liver vit. C reserves, it was considered desirable to determine whole blood ascorbic acid levels in chicks from each treatment. Leucocyte or whole blood vitamin C is considered by Butler and Cushman(14) to be a suitable measure of the state of vit. C nutrition. The results of these determinations also are presented in Table IV.

No changes were observed in total liver lipids of chicks fed 5,280 USP units of vit. A per kg of diet regardless of protein level, in spite of the fact that chicks fed the high protein diet showed liver stores of vit. A which were only 10% of the amount of vit. A in the livers of chicks fed the moderate protein diet. The amount of vit. A in the diet did not influence blood ascorbic acid levels appreciably. It appears, therefore, that in the chick, vit. A deficiency does not cause decreased ascorbic acid blood levels. The slight decreases in blood ascorbic acid levels as the dietary protein was increased, probably are a reflection of the need for ascorbic acid in the metabolism of certain amino acids, particularly phenylalanine and tyrosine(Sealock *et al.*, 15).

Experiment 3. In this experiment 2 extremely high levels of vit. A were fed to chicks by the equalized paired feeding technique, in order to conduct a study on the effects of protein upon apparent absorbability of vit. A. The experimental plan and results are shown in Table V.

These results showed that total liver vit. A and blood serum vit. A decreased as dietary protein increased. Vit. A content of the excreta (urine and feces combined) was accurately measurable only in the chicks fed 100,000 USP units of vit. A per kg of diet. The livers of the chicks fed the high protein diet contained 60% less, and 24-hour vit. A

TABLE V. Effect of Various Dietary Protein Levels upon Vit. A Storage and Excretion.

Treatment			Vitamin A				
					Excreta		
Protein level	Vit. A level, USP units/kg	4 wk wt, g	USP units per liver	USP units per cc blood serum	USP units per g (D.M. basis)	USP units per 24 hr (D.M. basis)	Apparent absorbability, %
High	440	189 (5/20)*	—	—	—	—	—
Mod. high	440	314 (15/20)	—	—	—	—	—
Moderate	440	288	—	—	—	—	—
High	50,000	275	6,723	2.30	—	—	—
Mod. high	50,000	284	11,249	2.64	—	—	—
Moderate	50,000	285	15,158	3.20	—	—	—
High	100,000	293	25,092	2.65	68	18,156	71
Mod. high	100,000	301	40,674	2.85	64	9,280	83
Moderate	100,000	300	63,369	3.71	75	6,000	89

* No. of chicks surviving over No. started. No mortality in the other lots.

excretion of these chicks was 200% greater than in the chicks fed the moderate protein diet. Since little if any intact vit. A is excreted in the urine(16), it appears, therefore, most likely that the higher than normal levels of protein interfere with absorbability of vit. A. Regardless of protein level, the amount of vit. A per gram of dry matter excreted was equivalent. However, the chicks receiving the high protein diets showed a very high total excretion, together with a markedly increased dry matter excretion. Therefore, total vit. A excreted by the chicks receiving the highest protein diet was much greater than that of chicks receiving the moderate protein diet.

During this experiment, a leg weakness was observed in many of the chicks receiving the high protein diets. At termination of the experiment tibias from chicks receiving the 2 highest vit. A levels were analyzed for bone ash. The results of these analyses indicated that the high protein levels interfered with calcification. Bone ash values were 36%, 40% and 43% for chicks receiving the high, moderately high and moderate protein diets, respectively. The reduced bone ash content of the tibias of chicks fed the high protein diets may indicate an interrelationship between dietary protein and vit. D or mineral metabolism in the chick.

Summary. 1. At a constant level of dietary vit. A, as the dietary protein was increased, vit. A content of the liver and blood serum of the chick decreased. Also in chicks receiving purified diets containing marginal amounts of vit. A and high protein levels, symptoms of vit. A deficiency occurred sooner and mortality was increased as compared to chicks fed diets containing moderate or low amounts of protein. 2. The decreased vit. A liver storage, resulting when the dietary protein was increased, could not be correlated with changes in liver lipids, nor vit. C blood levels. 3. The ataxia, mortality and other symptoms in young vit. A-deficient chicks was neither preceded nor necessarily accompanied by an increased blood uric acid level. A high protein diet produces high blood uric acid levels regardless of amount of vit. A in the diet. 4. Regardless of dietary vit. A level, the tibia bone ash of the chicks fed the high protein diets decreased as compared to that of chicks receiving a moderate level of protein in the diet.

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Effect of Therapeutic and Toxic Concentrations of Ouabain Upon Potassium Content of Myocardium.* (26427)

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In recent years, considerable attention has been devoted to the possibility that the actions of cardiac glycosides may be related to effects upon the electrolyte content of the myocardium. Several investigations(1-6) have indicated that a loss of potassium from the myocardium occurs in presence of toxic concentrations of these drugs. Other studies have demonstrated inhibition by cardiac glycosides of potassium uptake by red blood cells(7,8) and skeletal muscles(9). It is not clear, however, if the positive inotropic action of cardiac glycosides is also associated with a change in intracellular potassium concentration. We have, therefore, investigated the effect of both "therapeutic" and toxic concentrations of ouabain upon intracellular potassium content of isolated, contracting myocardium.

Methods. Ventricle strip preparation. Strips were prepared from the right ventricular wall of hearts of male guinea pigs (150-250 g) and stimulated at a frequency of 100/min. at 37.5°C. in a bicarbonate medium (pH 7.4) gassed with 1% CO_2-99% O_2 mixture as described previously(10).

Determination of extracellular space. Ventricle strips were equilibrated in medium containing 1% (w/v) inulin (dissolved by gentle heating) for the 3 hour experimental period. Inulin content of the blotted tissue was determined by the method of Ross and Mokotoff(11), adapted to approximately 50 mg of wet tissue, and extracellular (inulin) space then calculated.

Determination of potassium. At the conclusion of the experimental period, the ventricle strips were blotted with filter paper, weighed and total water determined by drying at 110°C. Each strip was then digested for 10 minutes with 1.5 ml of 0.75 *N* HNO_3 in a boiling water bath(3). The extracts were filtered and potassium determined with a Baird Atomic KY flame photometer employing an internal lithium standard. Intracellular potassium concentration was calculated from the determined values of total water, extracellular space, total potassium and potassium concentration of the medium. Intracellular potassium concentration was determined for each strip, using the corresponding mean extracellular space values for strips in the absence of drug or which had been exposed to ouabain at various concentrations.

Experimental procedure. Control strips were set up and stimulated for 3 hours, then extracellular space and potassium content were determined. Because of their small size, separate strips were used for determination of potassium and extracellular space. Other strips were stimulated for a 2-hour period, ouabain was then added and the strips were stimulated for an additional hour after which extracellular space and potassium content were measured.

Results. Ouabain at a concentration of 0.16 μg/ml produced a marked increase in the force of contraction without any evidence of toxicity (*e.g.*, spontaneous beats or increase in resting tension). Maximum increase in force (Table I) usually occurred within about one-half hour and was sustained during the remainder of the experimental period (one hour after addition of ouabain). A similar response occurred to a somewhat higher concentration of ouabain (0.33 μg/ml), although 3 of a total of 17 strips showed evidence of toxicity (Table I) at this concentration. With a consistently toxic concentration of the drug (1 μg/ml) there was initially a large increase in force of contraction to a mean value of about 110% of the initial force just prior to the point at which spontaneous contractions independent of the frequency of stimulation first appeared (the mean time to

* This investigation was supported in part by research grants from Nat. Heart Inst., P.H.S., and Los Angeles County Heart Assn.

TABLE I. Effects of Therapeutic and Toxic Concentrations of Ouabain upon Intracellular Potassium Concentration of Guinea Pig Ventricle Strips.

Ouabain conc., μg/ml	Increase* in force of contraction, %†	Evidence of toxicity	Total water	Extracellular space	Intracellular space	Total potassium, mmoles/kg wet wt†	Intracellular potassium conc., mmoles/l†
			ml/kg wet wt†				
0	—	None	780 ± 3 (12)‡	264 ± 6 (10)‡	516 ± 3	69 ± 1 (12)‡	131 ± 2
.16	52 ± 5 (20)‡	"	788 ± 8 (12)	277 ± 12 (8)	511 ± 8	66 ± 2 (12)	126 ± 2
.33	80 ± 8 (14)	" §	783 ± 6 (6)	275 ± 8 (8)	508 ± 6	65 ± 1 (6)	125 ± 2
1.0	See text (16)	Arrhythmia & increase in resting tension	804 ± 5 (8)	253 ± 12 (8)	551 ± 5	58 ± 1 (6)	103 ± 2

* Expressed as % of initial force. Initial force defined as steady force attained at end of 2 hr equilibration period (immediately prior to addition of drug).
† Mean value ± S.E.
‡ No. of experiments given in parentheses throughout table.
§ 3 strips at this concentration of ouabain displayed evidence of toxicity (spontaneous contractions of irregular force at a rate independent of frequency of stimulation, although no increase in resting tension occurred). Values given in the table are those for strips which did not show such evidence of toxicity at this concentration of ouabain.

appearance of first spontaneous contractions was 8 minutes and spontaneous frequency was about 250/min). The resting tension of the strips began to increase greatly at approximately the time of first appearance of spontaneous contractions, and after approximately an additional 5 minutes all visible contractile activity of the muscle ceased except for a slight quivering motion at a greatly increased resting tension. A concentration of ouabain of 0.16 μg/ml, therefore, produced a positive inotropic response such as that which occurs after therapeutic doses of such drugs, while a concentration of 1 μg/ml led to obvious evidence of toxicity.

The mean intracellular potassium concentration of the strips in presence of concentrations of ouabain which increased the force of contraction without evidence of toxicity (0.16 and 0.33 μg/ml) did not differ significantly from that of control strips ($P>0.1$, t test). On the other hand, a toxic concentration of ouabain (1.0 μg/ml) caused a significant decrease (21%) in intracellular potassium concentration by comparison with control strips ($P<0.01$). Intracellular space was not affected by ouabain at concentrations of 0.16 or 0.33 μg/ml, but was increased significantly ($P<0.01$) at a concentration of 1.0 μg/ml.

Discussion. The effect of therapeutic concentrations of cardiac glycosides upon potassium content of the heart has long been disputed, and a decrease, an increase or no change(12) have been reported. In many of the studies reporting a loss of this ion, however, clear evidence of a positive inotropic action without toxicity was lacking. Vick and Kahn(4) state that detectable losses of potassium from guinea pig hearts occurred with concentrations of ouabain producing a positive inotropic action. Their data showed a small and almost identical potassium loss over a wide range (10-75%) of positive inotropic action, but no statistical evaluation was presented. Later work(5) indicated that low concentrations of dihydro-ouabain, which produced a substantial positive inotropic action, resulted in at most a small loss of potassium for a brief period after which the ventricles were in potassium balance for several hours. Vick concluded that the positive inotropic action was not due to a loss in potassium, as several non-steroid lactones caused potassium loss without producing an increase in the force of contraction. Brown *et al.*(13) have shown recently that potassium loss occurred for a brief period only after injection of a cardiac glycoside into the circulation of heart-lung preparations in which cardiac failure had been induced with

pentobarbital. The significance of this fleeting effect with respect to the positive inotropic action is unknown. There is also no clear evidence in the work of Hajdu(3) that concentrations of cardiac glycosides producing increases in the force of contraction of frog heart without toxicity caused potassium loss, although concentrations producing contracture in 20-25 minutes did result in such loss. Loss of potassium occurring after exposure of guinea pig hearts to K-strophanthoside was followed within a few minutes by contracture(14).

Our studies indicate clearly that there is no significant change in intracellular potassium concentration or total potassium content in the presence of concentrations of ouabain producing a marked increase in the force of contraction but no evidence of toxicity. The view that an alteration in the potassium balance of the heart is related to the positive inotropic action is, therefore, untenable in our opinion unless loss of potassium from only certain regions of the heart occurred, in which case such loss might not have been detectable when total potassium was determined.

On the other hand, a significant decrease in intracellular potassium concentration and total potassium occurred in our experiments in presence of a toxic concentration of ouabain. Loss of potassium from cardiac tissue after toxic doses of cardiac glycosides has been reported by a number of other workers (1-6).

Summary. Intracellular concentration and total amount of potassium in isolated guinea pig ventricle strips in the presence of both therapeutic and toxic concentrations of ouabain have been investigated. A concentration of the drug which produced an increase in the force of contraction without evidence of toxicity did not alter either intracellular concentration or total amount of this ion in the heart. A toxic concentration of ouabain which produced automaticity and a marked increase in the resting tension, followed by decline in force of contraction to zero, caused a mean decrease of 21% in intracellular potassium concentration.

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Increased Concentration of Free Fatty Acids in Liver Disease.*† (26428)

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Plasma free fatty acids (FFA), although quantitatively small, may be an important form of fat transport from adipose tissue to other tissues for oxidative metabolism(2-4). The turnover is rapid(5,6) and plasma concentrations are affected by the nutritional state and certain hormones. Plasma FFA concentrations appear to be primarily regulated by the rate of their release from peripheral adipose tissue and have been correlated with factors affecting peripheral glucose or non-fat calorie utilization(4,7,8). The demonstration of decreased peripheral glucose utilization in cirrhosis(9), in addition to the decreased hepatic oxidation of fat noted in animals on protein deficient diets(10), has suggested that patients with cirrhosis may have abnormal FFA metabolism.

The response of plasma FFA to fasting in patients with acute and chronic liver disease is reported here.

Methods. Plasma FFA was determined by the method of Dole(2), and ketones by the method of Greenberg and Lester(11) as modified by Boshell *et al.*(12). All determinations were made after an 11-16 hour fast except for 3 patients with hepatic coma who were receiving I.V. glucose or oral carbohydrate at regular intervals throughout each 24 hours.

Plasma FFA concentrations were determined in a group of 27 male and 7 female alcoholics hospitalized with cirrhosis. This group comprised 20 patients considered to have chronic cirrhosis by clinical and laboratory evaluation and 14 with active disease, including 6 with jaundice, 3 with jaundice and hepatic coma or pre-coma, and 5 with episodic stupor (a chronic neuropsychiatric disorder associated with stable liver function, increased portal collateral circulation, and intermittent hyperammoniemia). Ascites was present in 7 patients in the chronic group and 7 of those with active disease.

Twenty-eight determinations of FFA concentration were made in 13 male and 15 female subjects without cirrhosis demonstrable by clinical or laboratory means, including 13 ambulatory normals and 15 patients hospitalized for other causes (acute alcoholism, biopsy-proven fatty liver, gastroenteritis, peptic ulcer, chronic bronchitis, pancreatic islet cell adenoma, epilepsy and idiopathic hirsutism).

Blood ketones were determined in 13 of the patients with cirrhosis (4 with jaundice) and in 13 without cirrhosis.

Results. Plasma FAA concentrations after 11-16 hours of fasting are shown in Fig. 1. FAA concentration in 28 subjects without cirrhosis was between 0.27 and 1.27 meq/l, with a mean of 0.73 meq/1 (± 0.04 S.E.M.). No correction was made for obesity or diet previous to the fast. Bierman *et al.*(13) reported plasma FAA concentrations of 0.57 meq/1 for normal fasting non-obese subjects and concentrations of 0.86 meq/1 for normal fasting obese subjects without glycosuria; however, the length of fast was not noted. Dole(2) lists normal overnight fasting FAA concentrations of 0.5-0.9 meq/1 and observed an increase with continued fasting.

Twenty patients with chronic cirrhosis of the alcoholic had FAA concentrations ranging from 0.46-1.13 with a mean of 0.88 meq/1 (±0.04 S.E.M.). The presence of ascites could not be correlated with FAA concentrations. Fourteen patients with active cirrhosis had concentrations ranging from 0.37-1.82 with a mean concentration of 1.27 meq/

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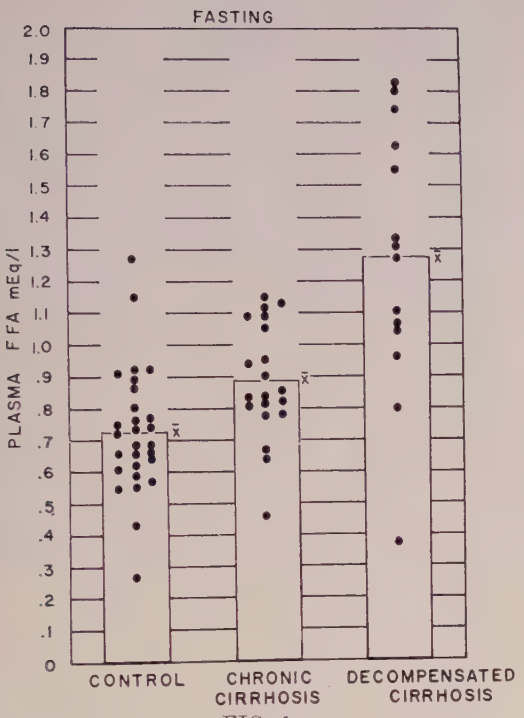

FIG. 1.

(±0.11 S.E.M.). Abnormally elevated as well as abnormally depressed FAA concentrations were found in the patients with jaundice. All patients with hepatic coma or episodic stupor had FAA concentrations above 0.9 meq/1 (Fig. 1). Comparison of the chronic and active cirrhosis patients (Student's t-test) revealed p value less than 0.001, indicating a high degree of significance of the differences between the mean values; although there is considerable overlap of the concentrations. (Fig. 1).

Blood ketone determinations ranged from 0.9 mg/100 ml to 3.7 mg/100 ml with a mean of 2.10 mg/100 ml (±0.27 S.E.M.) for 13 persons without cirrhosis while 13 patients with cirrhosis had concentrations ranging from 0.9-3.4 with a mean of 1.91 mg/100 ml (±0.36 S.E.M.). Four of these patients had jaundice and the concentration in these patients ranged from 0.9-3.4 with a mean of 1.70 mg/100 ml (Fig. 2). Recant(14), using the Greenberg-Lester method, reported a mean of 2.28 mg/100 ml (±0.47 S.E.M.) for non-hospitalized subjects without liver disease and 2.06 mg/100 ml (±0.47 S.E.M.) for hospitalized controls.. Mean concentration reported by Recant for patients with cirrhosis was 0.9 mg/100 ml (±0.22 S.E.M.) with a range of 0.00-2.08 mg/100 ml which was roughly correlated with severity of cirrhosis.

Discussion. Elevated plasma FFA concentrations have been reported in obesity(13), diabetic acidosis(13), hyperthyroidism(15), following prolonged fasting, after adrenalin and growth hormone injection(2,3,16) and in carbon tetrachloride poisoning in dogs(17). Conditions associated with decreased glucose utilization as well as those associated with loss of fat from fat depots appear to have elevated plasma FFA concentrations. The elevation of fasting FFA concentrations in active cirrhosis reported here is similar to levels described in diabetic ketosis(13) and suggests an abnormality in FFA metabolism and/or transport which may be related to the abnormal carbohydrate metabolism previously reported in acute hepatic failure and hepatic coma(9,18-20).

The lack of elevated blood ketone concentrations in patients with increased FFA concentrations is in accord with the results of Recant(14) who found lowered fasting blood ketone concentrations in patients with cirrhosis, roughly proportional to the severity of cirrhosis, and little or no measurable ketones in many patients with active cirrhosis. This may reflect further abnormalities of intermediary metabolism in cirrhosis (either decreased hepatic ketone production or increased peripheral utilization of fatty acids

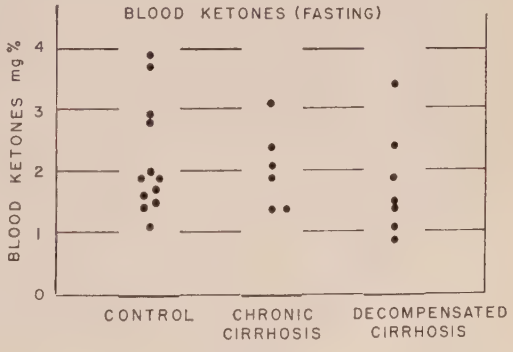

FIG. 2.

and ketone bodies). The hypoketonemic effect of adrenal cortical steroids(21) may be pertinent as cirrhosis patients have a defect in the metabolism of the steroid nucleus(22).

There is strong evidence to suggest that fatty acids in the form of FFA are directly transported from fat depots to other tissues where they may be used as substrate for oxidative metabolism(6). Plasma FFA have a rapid turnover and are transported bound to albumin and low density lipoproteins. As plasma FFA concentration appears to be primarily regulated by peripheral adipose tissue in response to alterations in peripheral nonfat calorie metabolism, the elevated fasting FFA levels and the reported abnormalities of glucose metabolism in cirrhosis would be consistent with a peripheral defect. Nevertheless, the demonstration of intrahepatic abnormalities in lipid synthesis and oxidation in animals fed alcohol or abnormal diets(10, 23) indicates that hepatic uptake and utilization must also be considered. Our results do not reveal an increased FFA concentration in acute alcoholics without cirrhosis or in nutritional fatty liver. The data reported here offer no evidence for determining the cause of the elevated plasma FFA concentrations in acute cirrhosis or hepatic coma. A recent report, while consonant with our observations, has suggested an hepatic defect(24).

Summary. Fasting plasma FFA concentrations were determined in 34 subjects with cirrhosis and 28 without cirrhosis.

Mean fasting FFA concentrations were elevated in subjects with cirrhosis and this was most marked in those subjects with acute hepatic failure or hepatic coma. The increased FFA concentrations were not accompanied by a rise in blood ketone concentrations.

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Influence of a Liver Preparation on Liver of Cats Receiving Carbon Tetrachloride. (26429)

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While lipotropic substances and proteins, especially those providing certain essential amino acids, exert a protective action on the liver, the participation of other unknown factors is suggested by clinical observations on patients with hepatic cirrhosis. That the liver may be the source of such unknown factors is implied in several studies. Beneficial effects of crude liver preparations have been reported in patients with decompensated cirrhosis of the liver who were maintained on either a moderate or high protein diet(1,2,3). Analyses of the available data of the various liver preparations indicate that their content of known lipotropic agents (*e.g.*, methionine and choline) was too small to account for the beneficial effects obtained over and above those obtained from the diet alone. A few experimental animal studies also suggest that liver extracts partially protect against experimental liver injury produced by dietary means or hepatotoxic agents(4,5,6,7). The results from animal studies, however, have been equivocal. In the present study an attempt has been made to evaluate by cytological quantitative determinations the influence of a lipotropic liver extract on the pattern and degree of fatty degeneration resulting from carbon tetrachloride.

Procedure. This study was conducted on 32 healthy adult male cats acclimatized by previous residence for 4 weeks in individual cages in a constant temperature room. The animals were maintained on a diet of fresh horse meat *ad lib* supplemented with commercial preparations of cat food (fish and chicken). Daily records of animal weight and food and water intake were kept. Daily food consumption was in each case far in excess of the calculated minimum requirements for both caloric and protein adequacy.

The 32 cats were divided into 4 groups: Group I, 9 cats: carbon tetrachloride* was given intramuscularly every second day, 0.8 g per kilo body weight, for 5 periods extending over 10 days. Group II, 7 cats: a liver preparation was given daily, 0.3 ml per kilo body weight, by deep intramuscular injection for a period of 20 days. Group III, 9 cats: the liver extract was given daily as in Group II but in addition carbon tetrachloride was given as in Group I from 11th to 20th days. Group IV, 8 cats: basal diet alone.

The liver preparation† (Ripason) used was a sterile protein-free water-soluble fraction processed from fresh untreated liver of cattle. It is represented by the manufacturer as containing the following per ml: cystine 105 μg, methionine 100 μg, histamine 2.0 μg, and vit. B_{12} less than 0.01 μg. In terms of per cent of dry residue, total N was 6.2, amino N 2.3, total P 2.1, and amino acids 0.6.

The animals were killed rapidly by overdosage with pentobarbital sodium by intracardiac puncture. Sections of 5μ thickness were made from each of 12 uniformly distributed areas of the liver. Degree of liver damage was determined by a fat droplet count procedure similar to that described by Tanyol and Rehfuss(8). Microscope slides of liver sections were examined under power of 5 $\times$ ocular and 4mm objective. The size of the examination field was reduced uniformly to a circle 6.5mm in diameter. A count in 20 consecutive fields was made of the fat droplets seen in each slide and 3 consecutive slides obtained from each area by serial section were used. A total of 60 fields for each area of liver was thus examined. Areas which were within the field but which did not contain liver cells (*e.g.*, periportal spaces, larger vessels, parts severed from main section, etc.) were not included in the count.

The data on fat droplet counts from the 4

* Tetrachlormethane reagent, sp.gr. 1.59.

† The authors are indebted to Robapharm Laboratories A G, Basel, Switzerland, for the preparation.

TABLE I. Influence of Administration of a Liver Preparation on Hepatotoxic Effect of Carbon Tetrachloride.

Treatment	Animal No.	Fat droplets, mean count per 20 fields
Group I:		
Carbon tetrachloride, alternate days for 10 days	1	16
	2	64
	3	461
	4	198
	5	802
	6	532
	42-0	60
	42-1	16
	42-2	0
	N = 9	
Group II:		
Liver preparation daily for 20 days	14	0
	15	0
	16	16
	17	16
	42-6	0
	42-7	0
	42-8	0
	N = 7	
Group III:		
Carbon tetrachloride, alternate days for 10 days plus liver preparation daily for 20 days	7	0
	8	0
	9	0
	10	20
	11	16
	12	168
	42-3	13
	42-4	0
	42-5	0
	N = 9	
Group IV:		
Diet alone, untreated	C 1	0
	C 2	0
	C 3	0
	C 4	0
	D 5	10
	D 6	0
	D 7	0
	D 8	0
	N = 8	

series of experiments were tabulated and significance of differences between them tested by the ranking method described by Wilcoxon(9).

Results. The appetites of the animals of Group II (liver extract) and Group IV (basal diet controls) were well maintained. Group I (carbon tetrachloride) and Group III (carbon tetrachloride plus liver extract) cats showed about a 10% decrease in food intake only during the last 2 days. In all animals of the 4 groups body weights showed no net decrease throughout the experiment. Other than an increasing resistance to needle injections, the animals showed no behaviour peculiarities during the observation period. No evidence of inflammatory reaction was found at sites of liver extract injections.

In general, the livers of the cats that received carbon tetrachloride (Group I) appeared on gross inspection to be slightly paler and more granular than those of non-intoxicated animals (Groups II and IV) but the differences could not be regarded as marked. Microscopic examination of the liver revealed fatty infiltration of all liver lobes in 8 of the 9 animals. Fat droplets were distributed diffusely rather than concentrated in any one region. Data on fat droplet count are summarized in Table I.

On the other hand, the microscopic picture of the livers of cats treated with both carbon tetrachloride and the liver extract (Group III) was different from the above. Fatty changes were marked in one cat, slight in 3, and not demonstrable in the remaining 5 (Table I). The cats treated with the liver preparation had significantly smaller numbers of fat droplets than the untreated animals, with a probability of rank T less than 0.05%(9). Fig. 1 and 2 show sections typical for livers of cats receiving carbon tetrachloride alone, and of cats receiving carbon tetrachloride plus liver extract.

While the usual signs of nuclear degeneration (karyolsis, karyorrhexis and pyknosis) and cytoplasmic degeneration (vacuolization and loss of cell contour) were much more marked in livers of intoxicated cats, their absence in the liver extract treated series can not be regarded as specific to the treatment.

Discussion. All evidence of hepatic damage due to carbon tetrachloride may disappear spontaneously if the toxic agent is withdrawn (and animal is maintained on a stock diet)(10,11,12). In the present experiments, a low incidence of hepatic damage during carbon tetrachloride intoxication was found only when associated with administration of the liver preparation. Whether this represented stages in regression of established lesions or reflected prophylactic properties of the liver extract we do not know.

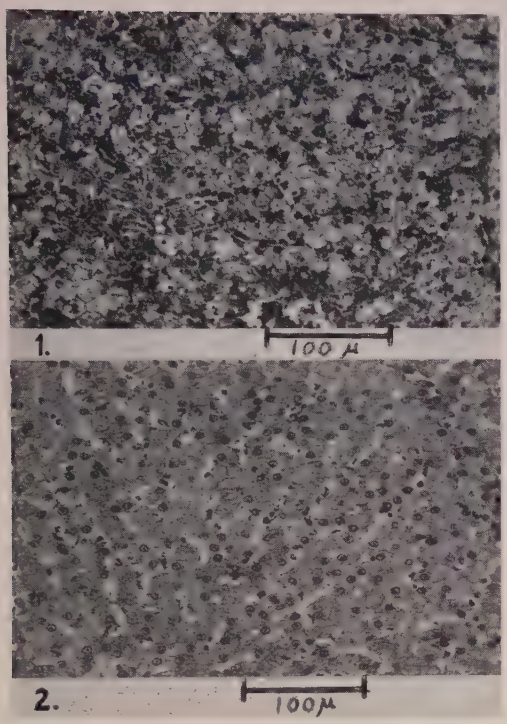

FIG. 1. Section of liver of cat which received intramuscularly 5 evenly spaced doses of carbon tetrachloride over a 10-day period.

FIG. 2. Section of liver of cat which received a protein-free water soluble liver extract daily in addition to carbon tetrachloride over a 10-day period.

In hepatic injury removal of lipid from the liver cells can be brought about only when the diet provides lipotropic factors, principally supplementary methionine, choline, or a choline precursor. In addition the supply of dietary protein must be adequate to provide the essential amino acids necessary as structural materials for reparative processes in the liver cells(13). The effectiveness of the liver preparation in these experiments probably was not due to simple provision of a lipotropic agent since the amounts of choline and methionine were far below the minimum requirements. Furthermore, prevention by methionine or choline supplements of hepatoxic effects of either carbon tetrachloride(14,15) or chloroform(16) is not observed in the rat, cat, or dog maintained on a high protein intake. The ameliorating effects of the liver preparation would also appear not to have been due to its content of Vit. B_{12} since the amount of Vit. B_{12} so administered was less than .003 μg/kilo above that provided by the stock diet.

Protection of the rat liver against hepatatoxic agents by suspensions of tissue(17), xanthine compounds(18,19), and even colloidal carbon(20) have been reported. This effect has been interpreted as being due to increased body tissue catabolism incident to inflammatory reactions at injection site(20). In the present experiments, however, there were no inflammatory reactions noted. Moreover, in another study(16) we have repeatedly failed to find in carbon-tetrachloride intoxicated cats any protection against liver damage from similarly administered protein-free water-soluble preparations of heart muscle or stomach tissue. The evidence supports the view that the beneficial effects of the liver extract were not the result of a non-specific reaction but rather due to a lipotropic factor. More definite chemical characterization of the lipotropic factor(s) must await further purification of active liver fractions.

The absence of an injury pattern would appear not to support the view that different degrees of fatty and other degenerative changes reflect stages in a progressive invasion from the central vein outward until the whole lobule is involved. Finally it should be noted that none of the liver sections from our relatively short-term experiments demonstrated the presence of fibrous tissue or reticulum seen in liver biopsy specimens from patients with hepatic cirrhosis. The report that certain liver extracts inhibit growth and migration of fibroblasts(21) suggests the study of the effectiveness of the liver preparation used by us in chronic hepatic injury by toxic agents.

Summary. Diffuse fatty infiltration of all liver lobes was found in cats given carbon tetrachloride intramuscularly. A statistically significant reduction in degree of fatty infiltration was found when the cats were also given injections of a protein-free aqueous fraction of liver. The nature of the lipotropic action is unknown.

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Distinctive Cytopathology of ECHO Viruses Types 22 and 23.*† (26430)

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The cytopathology of the enteroviruses in rhesus monkey kidney tissue culture has been described(1). All of the enteroviruses studied (poliovirus 1-3, Coxsackie B1-B5, A9, ECHO 1-14) manifested a characteristic cytopathic effect (CPE) in fixed and stained preparations. Only ECHO virus type 10 (Reovirus type 1) was found to have features which differed from the other enteroviruses. In the present paper, the study was extended to ECHO serotypes 15-24. Types 22 and 23 were found to have distinctive CPE. The cytopathology of the remainder was similar to that previously described for the members of the enterovirus group.

Materials and methods. Tissue cultures. Rhesus monkey kidney coverslip cultures were prepared as described previously(1). Cultures were maintained in 1.8 ml of a medium consisting of 0.5% lactalbumin hydrolysate, 0.1% yeast extract (Difco), 0.2% bovine albumin, Fraction V (Armour and Co.) in a base of Earle's saline with 200 units penicillin, 200 μg streptomycin per ml and inoculated with 0.2 ml undiluted virus§ suspension. Titers of ECHO 22 and 23 were 5.1 and 5.3 log TCD_{50}/0.1 ml respectively. At selected intervals, coverslips were removed and fixed for staining. HeLa cell cultures grown in a medium containing calf serum were also used(3).

Histologic technic. Hematoxylin and eosin stain as modified by Reissig was used routinely(4). Feulgen stain was also used(5). Cultures were fluorochromed with acridine orange at pH 6.0(6) and examined under the fluorescence microscope. The fluorescent equipment consisted of a Reichert 'Lux X' high intensity mercury vapor arc lamp, 2 BG12 ultraviolet filters and a modified model

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‡ Markle Scholar in Medical Science.

§ ECHO virus prototypes were obtained through the courtesy of members of the Committee on Enteroviruses (2).

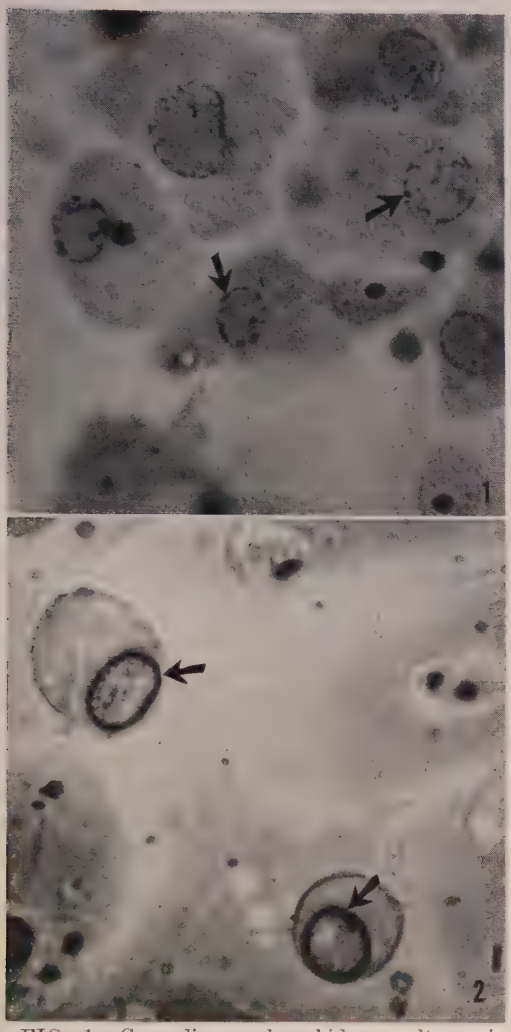

FIG. 1. Coverslip monkey kidney cultures infected with ECHO 22 and stained with hematoxylin-eosin. Cells show rounding loss of nucleoli with peripheral beading of chromatin. Photographed under oil immersion, ×970.

FIG. 2. Coverslip monkey kidney tissue culture infected with ECHO 22 and stained with hematoxylin-eosin. Two cells show 'empty' nuclei with prominent nuclear membranes. Photographed under oil immersion, ×970.

No. 414 American Optical binocular microscope with 35 mm camera attachment. With this equipment, observations under oil immersion (970×) were satisfactory.

Results. Examination of infected cultures stained with hematoxylin and eosin revealed that, with the exception of types 22 and 23, the CPE of ECHO viruses types 15-24 was characteristic of the enterovirus group(1). The typical enterovirus cytopathic changes consisted of rounding of cells, formation of a round, eosinophilic cytoplasmic mass and enlargement of eosinophilic nuclear granules. The cytoplasmic mass indented or appeared to cause folding of the nucleus. Formation of basophilic cytoplasmic granules was observed in late stages of infection. The changes occurring in the cytoplasm of cells infected with ECHO 22 and 23 were similar to those seen with other enteroviruses. The major differentiating features occurred in the nuclei. Since the cytopathic changes were the same for the prototype ECHO 22 and 23 strains, ECHO 22 prototype was selected for more detailed examination. Studies with ECHO 22 revealed a sequence of changes appearing in the following order: 1) *Rounding of cells and appearance of an eosinophilic cytoplasmic mass with no nuclear changes.* The nuclei remained oval or round and the cytoplasmic mass was present in all subsequent stages. 2) *Decrease in staining intensity of the nucleolus.* In this stage, the nucleolus stained very faintly giving a faded appearance. 3) *Disappearance of nucleolus without changes in chromatin.* 4) *Beading of nuclear chromatin either diffusely or peripherally.* (Fig. 1). In this stage, the chromatin of the nucleus appeared to be in dense clumps or 'beads' which were either arranged around the inner surface of the nuclear membrane or scattered diffusely throughout the nucleus. 5) *Disappearance of chromatin leaving an 'empty' appearing nucleus.* (Fig. 2). In this stage, the nucleus appeared to be devoid of any chromatin and the nuclear membrane was smooth and stained intensely. There was, however, in some nuclei, a very faintly staining, fine reticular network.

At selected intervals, 100 cells in stained preparations were examined and the number of these cells manifesting CPE was tabulated (Fig. 3). Throughout the period studied, relatively few cells showed CPE. Changes in cells appeared at 8 hours and the number of these cells increased through 12 hours and then decreased. The number of cells involved did not exceed 28% of total number of cells in the culture.

Nuclear changes at the various stages of infection have been summarized in Table I.

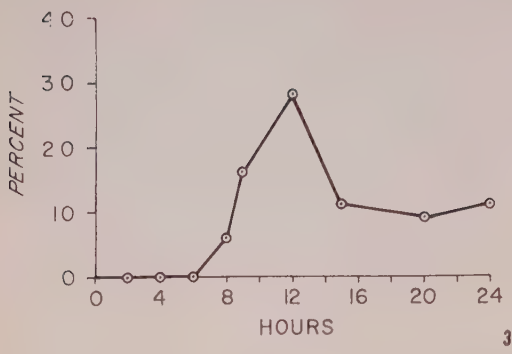

FIG. 3. Involvement of cells in fixed and stained monkey kidney tissue culture inoculated with ECHO 22. Percent = No. of cells manifesting CPE/100 cells.

The numbers were obtained by examining 100 cells manifesting CPE at times indicated. Rounded cells with an eosinophilic cytoplasmic mass and no nuclear changes were most numerous at 8 hours. Nuclei, which showed decrease in staining intensity or 'fading' of the nucleoli, sharply increased in number from 8 hours through 9 and 12 hours and then decreased. Nuclei with absent nucleoli but with no chromatin changes gradually increased in number from 8 hours through 20 hours and then decreased. Nuclei with absent nucleoli and with beaded chromatin appeared at 9 hours in small numbers, gradually increased through 20 hours and then showed a slight decrease. Cells with so-called 'empty' nuclei showed a steady increase from 9 hours through 24 hours. All cells showing CPE were rounded and had an eosinophilic cytoplasmic mass.

In wet preparations of cultures, cytopathic changes, which consisted of rounding and increased refractility of cells, generally appeared first at the edges of the monolayer and later spread throughout the culture. Neutralization tests and virus titrations were easier to read microscopically when the cultures were incubated on a roller drum. In fixed and stained HeLa cell cultures, ECHO 22 manifested a CPE similar to that seen in fixed and stained monkey kidney tissue culture.

Acridine orange fluorescence stain. Uninoculated cultures stained with acridine orange and examined under the fluorescence microscope showed a bright green nucleus indicative of DNA content and bright orange or orange-red cytoplasm and nucleoli indicating RNA content(6,7). In cells infected with ECHO 22 or 23, the thickened nuclear membranes fluoresced a bright green and the chromatin also appeared as bright green clumps. These structures were also Feulgen positive. Cytoplasmic masses were greenish and peripheral cytoplasm red or orange-red. In the 'empty' nuclei, there were fine reticular networks which fluoresced a faint gray-green and were Feulgen negative. There were no Feulgen positive structures in the so-called 'empty' nuclei.

Additional strains of ECHO 22. ECHO 22 has only recently been classified as a distinct serotype in the ECHO virus group(8). The role of this virus in human illness has not been defined. The prototype Harris strain was isolated in Ohio from a case of diarrhea by Dr. A. B. Sabin and his colleagues. ECHO 22 was isolated in Buffalo during a study of well infants in 1957 and also from clinical specimens submitted for virus diagnosis in succeeding years. The history of the Buffalo ECHO 22 strains has been summarized in Table II. The cytopathic effect of these strains in monkey kidney tissue culture revealed the same cellular changes as noted for the prototype Harris strain.

Discussion. The distinctive cytopathic changes resulting from infection with ECHO viruses types 22 and 23 were confined to the nucleus. The nucleus was round or oval and

TABLE I. Sequence of Nuclear Changes with ECHO 22.

Hr	No change	Faded nucleoli	No nucleolus	Beaded chromatin	'Empty'
0	0	0	0	0	0
2	0	0	0	0	0
4	0	0	0	0	0
6	0	0	0	0	0
8	83*	12	5	0	0
9	36	45	11	4	4
12	38	47	9	3	3
15	31	26	16	11	16
20	29	15	22	19	15
24	18	23	16	15	28

* No. of cells with nuclear changes, based upon count of 100 cells manifesting CPE.

TABLE II. History of ECHO 22 Strains.

Year	Strain	Clinical history	Age	Specimen
1957	Johnson	Well infant	2 mo	Rectal swab
1957	Host	" "	6 "	" "
1957	Morrison	" "	9 "	" "
1957	Borowiec	" "	15 "	" "
1957	Estelle	Diarrhea & jaundice	1 "	" "
1958	Hubbard	Diarrhea	5 wk	Throat swab
1960	Albaralla	"	13 mo	Stool

in the late stages, the nuclear membrane was prominent. The nucleoli and chromatin gradually disappeared leaving the so-called 'empty' nucleus. In contrast, cells infected with other enteroviruses showed indented or folded nuclei and unaltered nuclear membranes. The nucleoli and chromatin persisted until the late stages and nuclear granules became enlarged and more eosinophilic. Basophilic cytoplasmic granules observed in classical enterovirus type infection were absent in cultures infected with ECHO virus type 22 or 23.

In the experiments described, the multiplicity of infection was not controlled and the sequence of cellular changes was made on the basis of relative numbers of cells showing changes. The number of cells which appeared infected on the basis of cytopathology at any one time was found to be less than 30%. After 12 hours incubation total number of cells involved decreased (Fig. 3). The data to explain this phenomenon are not available from the present experiments.

The CPE in cultures infected with ECHO virus type 10 was distinguishable from either the typical enterovirus CPE or the changes described above for ECHO 22 and 23(1,9, 10,11). The main differentiating feature was the appearance of globular or filamentous eosinophilic material in the cytoplasm around or adjacent to the nucleus. ECHO 10 has been reclassified as Reovirus type 1(11) and one of the contributing features was the characteristic cytopathic effect in cell cultures. The use of cytopathology in establishment of the Reovirus group suggests that further studies of the physical and biologic characteristics of ECHO 22 and 23 should be undertaken to establish the relationships of these serotypes to the ECHO virus group and other enteroviruses.

The distinct cytopathic effect of viruses in tissue culture has proved to be a useful tool in identification of viruses isolated from the human alimentary tract. In our laboratory, using CPE as a guide, 7 strains of ECHO 22 were detected and confirmed serologically. Four strains were isolated from healthy infants and the remaining 3 were recovered from infants with diarrhea. ECHO 22 was isolated in this study only from very young children. Other viruses, such as adenoviruses, have been excluded from routine enterovirus serologic tests because of their cytopathology. Conversely, unknown agents have been assigned to the enterovirus antibody screening program when their cytopathology was found to resemble that of the enteroviruses.

At present there are no *positive* criteria for inclusion of an unknown virus into the enterovirus group. The results of this study indicate the use of cytopathology as a classification tool and it might be suggested that new agents considered for addition to the enterovirus group, particularly ECHO viruses, be examined from this point of view.

Summary. The cytopathology of ECHO 22 and 23 was compared with other enteroviruses and found to be distinctive. The important differentiating features appeared in the nucleus. An eventual disappearance of the nucleolus and nuclear chromatin and a thickening and increase in staining intensity of the nuclear membrane resulted in appearance of an 'empty' nucleus. Seven isolates of ECHO 22 were found to have similar cytopathic changes. It is suggested that detailed study of the cytopathic effect of viruses in tissue culture may play an important role in relating new agents to the enterovirus group.

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Binding of Calcium to Bence-Jones Proteins.* (26431)

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Bence-Jones proteins appear in the urine of approximately half of the patients who have multiple myeloma. The fact that these abnormal proteins of small molecular weight are barely detectable in blood signifies that they are efficiently and rapidly transferred from plasma to urine, and in some cases several grams may be excreted per day. Because patients with multiple myeloma frequently have bone lesions, the calcium binding properties of their pathological proteins are of interest. Prior investigations have revealed no special affinity of serum myeloma globulins for calcium(1,2), but the calcium ion binding properties of urinary Bence-Jones proteins have not previously been reported.

Materials and methods. In the present study, Bence-Jones proteins were obtained from the urine of 7 patients† having multiple myeloma by precipitation in ⅔ saturated ammonium sulfate at 4°C. The precipitates were dialyzed exhaustively against distilled water, and the resulting solutions were filtered into flasks and lyophylized. One of the proteins, that from patient C. P., used in these investigations was further purified by column chromatography of a solution of the lyophylized powder on DEAE-SF‡. Just prior to measurements of calcium binding, the lyophylized proteins were dissolved in water, then electrodialyzed in a collodion cell for 24 to 48 hours against flowing distilled water. Standardized calcium hydroxide was then added to effect the desired pH, followed by addition of calcium chloride to reach the desired Ca^{++} concentration. Total calcium values were derived from the measured volumes added. Calcium ion activities were determined by potentiometric measurements using a permselective sulfonated polystyrene-collodion membrane as described by Carr(3). Protein concentrations were determined by weight after drying at 105°C.

Ultracentrifugal analysis of all of the proteins confirmed their classification as Bence-Jones proteins on the basis of sedimentation constants of approximately 3S. Double diffusion in agar gel plates gave precipitin bands in combination with rabbit anti gamma globulin. Electrophoresis by moving boundary and starch gel zone methods indicated varying degrees of heterogeneity with the exception of one homogeneous Bence-Jones protein, C.P., which also was eluted by increasing phosphate, decreasing pH gradient as a

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† Bone lesions observed by roentgenography: osteolytic lesions, osteoporosis, and vertebral compression in one patient; osteolytic lesions and vertebral compression in one patient; only osteolytic lesions in one patient; only osteoporosis in one patient; and no demonstrable abnormalities in 3 patients.

‡ DEAE-SF: diethylaminoethylcellulose.

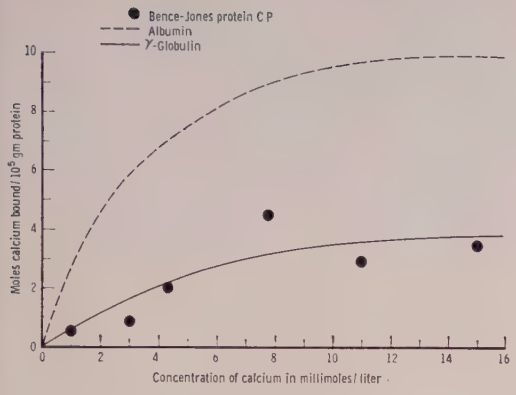

FIG. 1. Binding of calcium to Bence-Jones protein C.P. pH = 7.4. (The drawn curves represent previous results for blood proteins, conc. = 2%; the points are for Bence-Jones protein, conc. = 3.05%.)

homogeneous symmetrical peak from a DEAE-SF column.

Results. Experiments with calcium and Bence-Jones protein C.P. were carried out at pH 7.4 to test the effect of increasing concentrations of calcium ion. The results are summarized in Fig. 1. The solid line represents binding of calcium to gamma globulin previously reported by Carr(4), and the dots illustrate the data for Bence-Jones protein C.P. Binding of calcium with human serum albumin is indicated by the dashed line(4). The Bence-Jones protein, like gamma globulin, shows a comparatively low binding capacity for calcium at pH 7.4. Neither gamma globulins nor Bence-Jones proteins bind calcium to nearly the degree that serum albumin does. In another series of measurements on a different Bence-Jones protein (H.Z.), no detectable binding at pH 7.4 was found, even in a 3% protein solution at a calcium ion concentration of 15 mM/l.

The effect of pH on binding of calcium to the Bence-Jones protein C.P. is shown in Table I. The pH effect appears to be similar to that reported for other proteins(3,4). Calcium binding is higher at pH 10 and decreases progressively until it approaches zero at pH 6.0.

TABLE I. Binding of Calcium by Bence-Jones Protein C.P. at Several pH Values. Protein concentration = 1.69%.

pH	Added Ca	Measured Ca	Bound Ca	Bound Ca, moles/10^5 g protein
	mmoles/l			
10	4.64	3.23	1.41	8.34
8.9	4.64	3.38	1.26	7.50
7.9	4.64	3.71	.93	5.50
7.0	4.64	4.20	.44	1.63
6.0	4.64	4.62	.02	.12

Bence-Jones protein from patient H.Z. which was slightly heterogeneous electrophoretically possessed a very low calcium binding capacity. There was a slight though significant degree of binding at pH 8.8 (3.8 moles/10^5 g), but at pH 8 binding was only half as great, and at pH 7.4 there was no detectable binding. Three additional Bence-Jones proteins studied at pH 7.4 gave results similar to protein H.Z., whereas 2 additional Bence-Jones proteins indicated slight degrees of calcium binding similar to protein C.P. illustrated in Table I. There was no correlation between the severity of bone lesions in the patients studied and the extent to which calcium was bound by their Bence-Jones proteins.

Discussion. All of the data indicate that calcium is not very strongly bound by Bence-Jones proteins. The proteins with the highest affinities for calcium among those studied had binding capacities no greater than those reported for gamma globulin, a protein which has a low capacity for binding divalent cations. These proteins bind calcium to a much smaller extent than does serum albumin.

Carr and Woods(5) have shown that binding of magnesium to a variety of proteins was quantitatively similar in each case to binding of calcium, and further studies demonstrating competitive binding of these 2 ions to serum albumin indicated that binding sites on the protein common for both ions are involved(6). It is very probable that magnesium, as well as calcium, is only slightly bound by Bence-Jones proteins.

It is evident from these results that bone destruction frequently associated with plasma cell proliferation in patients who have multiple myeloma cannot be due to a direct chemical interaction of bone calcium with the Bence-Jones proteins which are believed to arise *de novo* in malignant plasma cells. The "punched out lesions" evident on roentgenographic examination of the bones of

these patients are probably due to lytic factors created by proximate proliferative activity of tumor tissue. It is possible that the generalized osteoporosis occurring in other patients may be due to these local factors operating in a more uniformly disseminated form of the disease, while the lack of bone lesions in still other patients with multiple myeloma may signify a less advanced stage of tumor infiltration.

Summary. Determinations of calcium ion activities by potentiometric measurements using permselective sulfonated polystyrene-collodion membranes indicate that calcium is not very strongly bound by Bence-Jones protein. It is concluded that bone destruction frequently associated with plasma cell proliferation in patients who have multiple myeloma cannot be due to a direct chemical interaction of bone calcium with the Bence-Jones proteins.

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Antibody Response in Hematologic Patients. (26432)

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Previous studies from this laboratory(1) demonstrated that antibody response to subcutaneous injection of phenolized tularemia vaccine in 92 of 105 splenectomized patients was similar to that observed in normal controls. Failure of the remaining 13 patients to exhibit antibodies was attributed to underlying disease rather than absence of spleen. As a correlative study, antibody response of patients with hematologic diseases and intact spleens was studied. Killed tularemia vaccine (Foshay) was employed because the incidence of naturally-occurring tularemia in this area is low, and the vaccine is not generally available. Thus it could be assumed that any response would be due to a primary antigenic stimulus.

Materials and methods. The subjects used in the study were selected at random from patients visiting the hematology clinics at University Hospital. "Normal" controls were selected from presumably healthy volunteers (medical students and employees). All received a single 0.5 ml subcutaneous injection of Foshay killed tularemia vaccine. Blood for serologic studies was obtained before immunization and 3-6 weeks after. Antibody titers were determined by the bacterial agglutination test described earlier(1).

Results. The response of 162 hematologic patients and 48 control subjects to a single injection of tularemia vaccine is presented in Table I. Antibody titers varying from 1:20 to 1:640 were detected in 45 of 48 (93.7%) controls. In contrast (Table I) only 67 of 162 (41.4%) patients with hematologic disorders showed agglutination titers varying from 1:10 to 1:640. The lowest incidence of antibody response was seen in chronic lymphatic leukemia where only 2 of 25 (8.0%) had titers of 1:10 and 1:640, respectively. Similarly only 5 of 20 (25.0%) lymphosarcoma patients showed titers varying from 1:10 to 1:320. A total of 15 of 37 (40.5%) Hodgkin's patients exhibited titers from 1:10 to 1:640. In the remaining smaller groups (Table I) antibodies were detected in 13 of 19 (68.4%) pernicious anemia and 5 of 12 (41.7%) chronic myeloid

TABLE I. Antibody Response of Hematologic Patients to Tularemia Vaccine.

Diagnostic group	No. of subjects	Sex ♂	♀
Hodgkin's disease	15/37*	12/21	3/16
Lymphosarcoma	5/20	3/9	2/11
Chronic lymphatic leukemia	2/25	2/15	0/10
Acute " "	2/3	0/1	2/2
" monocytic "	3/7	0/4	3/3
Chronic myeloid "	5/12	3/5	2/7
Polycythemia rubra vera	4/5	2/3	2/2
Pernicious anemia	13/19	4/6	9/13
Iron deficiency "	2/5	—	2/5
Aplastic "	3/5	0/1	3/4
Hemolytic "	0/2	0/1	0/1
Multiple myeloma	3/6	2/3	1/3
Reticulum cell sarcoma	1/2	1/1	0/1
Myelofibrosis	2/3	0/1	2/2
Hemochromatosis	1/2	1/2	—
Miscellaneous†	6/9	3/6	3/3
Total patients	67/162	33/79	34/83
Normal subjects	45/48	24/27	21/21

* Numerator = No. showing antibodies. Denominator = Total No. vaccinated.

† Antibodies formed: Achrestic anemia, giant follicular cell lymphoblastoma, thrombocytopenia purpura, allergic purpura, disseminated lupus erythematosis with sickle cell trait, thrombocytosis. No antibodies: Sickle cell anemia, hypoplastic anemia, reticulum cell leukemia.

leukemia patients in titers of 1:20 to 1:640. Results were variable in the remaining 11 disease category groups consisting of 49 patients in which only 27 (55.1%) exhibited antibodies.

There was no good evidence that antibody response was adversely affected by the various forms of treatment. For example, only 1 of 8 chronic lymphatic leukemia patients in remission and receiving no therapy exhibited antibodies. Similarly only 11 of 23 in the lymphosarcoma-Hodgkin's group not receiving therapy showed detectable antibodies.

Analysis of the serologic data in relation to age of patients which ranged from 13 to 82 years, revealed no association between age and antibody production. However there was an association between sex and antibody production in the lymphosarcoma-Hodgkin's group wherein 15 of 30 males as compared to only 5 of 27 females demonstrated antibodies. It is also of interest that the 2 of 25 showing antibodies in the chronic lymphatic leukemia group were males.

Discussion. To assess further the role of the spleen in antibody formation, patients with similar disease processes and intact spleens should be studied as previously described(1). However, since splenectomy is usually performed in this institution when various manifestations of hypersplenism are evident, such patients were not available. Since it was felt that the underlying disease rather than absence of the spleen was responsible for lack of antibody response in 13 of 105 splenectomized patients, other patients with blood dyscrasias were studied. The antibody response in hematologic disorders has been reviewed(2). The results obtained in our study are in agreement with the earlier reports(2-5) that antibody formation in chronic lymphatic leukemia is poor. There has not been general agreement on antibody response in Hodgkin's disease(4-9). Our studies demonstrated that in Hodgkin's and lymphosarcomas antibody response to a single injection of tularemia vaccine was detectable in only 20 of 57 patients. The numbers of persons studied in each of the other disease categories were too small to make definite conclusions other than that as a group, patients with hematologic disease processes did not form antibodies as well as normal individuals. Under the conditions of this study a sub-optimal single antigenic stimulus was used rather than the 3 daily injections routinely employed with Foshay vaccine. However, the difference between these 2 regimes was not great in our previous studies in which 39 of 48 (81.3%) and 52 of 57 (91.2%) demonstrated antibodies following 1 and 3 vaccine injections, respectively.

Of additional interest is the observation that only 5 of 27 female patients with lymphomas showed antibodies as compared to 15 of 30 males. Previous studies(1) from this laboratory in splenectomized persons showed that 11 of 70 females as compared to 2 of 35 males showed no antibody response after immunization. Also, properdin levels(10) were significantly lower in females than males. It is also of interest that the 2 of 25 patients showing antibodies in the chronic lymphatic leukemia group in this study were males. Further studies to elucidate these factors are in progress.

Summary. A single injection of 0.5 ml of phenolized tularemia vaccine elicited detectable agglutinating antibodies in 67 of 162 (41.4%) patients with hematologic disorders as compared to 45 of 48 (93.7%) in normal controls. Particularly significant was the lack of response in 23 of 25 chronic lymphatic leukemia and in 37 of 57 lymphoma patients. In the latter group only 5 of 27 females as compared to 15 of 30 males exhibited antibodies.

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Acute and Subacute Toxicity of Amino-Hexose-Reductones. (26433)

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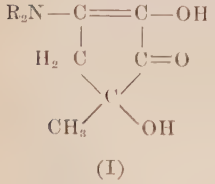
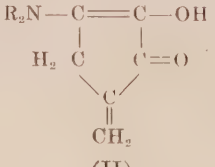
The well-known Maillard reaction, responsible for browning discoloration in many foods, involves combination of reducing sugars with amino groups of amino acids, peptides, and proteins, with subsequent enolization and dehydration of the sugar radical(1, 2). In studies on the sugar rearrangement and dehydration reactions, Hodge and collaborators(3,4) isolated new "amino-hexose-reductones," $R_2N \cdot C_6H_7O_3$ (I), and "anhydro-amino-hexose-reductones," $R_2N \cdot C_6H_5O_2$ (II), from reactions of hexoses with secondary amine salts and determined their structure(4). Because these reductones showed excellent antioxidant properties in fats and oils(5,6), toxicity studies were undertaken to determine their suitability for food use.



(I)



(II)

The present report presents data on the acute and subacute toxicity of 5 reductones, namely, dimethylamino - hexose - reductone (DMA) and its anhydro derivative (ADMA), piperidino-hexose-reductone (PIP) and its anhydro derivative (APIP), and morpholino-hexose-reductone (MORPH).

Methods. Aqueous propylene glycol was used as solvent for DMA and MORPH in acute toxicity studies employing oral and intraperitoneal administration. Because of lack of a suitable solvent of low toxicity for PIP, APIP, and ADMA toxicity determination of these reductones was limited to single oral administration of a suspension in 20% gum acacia. The limited amount of material available for determination of acute toxicity necessitated the use of mice (weighing 15 to 20 g each) except in the case of DMA for which an approximate determination of the toxicity was made by single intraperitoneal injection in rats (weighing 125 to 150 g) as well. In addition 2 groups of 5 mice each were given daily oral doses of 25

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and 50 mg/kg of DMA respectively for a period of 3 weeks, Saturdays and Sundays excluded, for detection of cumulative effects. All dosages refer to mg/kg of body weight.

A more severe test for detection of cumulative effects of each of the 5 reductones was a 100-day feeding experiment. Each reductone was studied by placing 5 young rats of each sex on dietary levels of 0.0, 0.06, 0.12, 0.25, 0.5, and 1.0% reductone in a basal diet having the following percentage composition: corn meal 73, casein 10, linseed oil cake meal 10, alfalfa 2, cod-liver oil 3, bone ash 1.5, and sodium chloride 0.5. The rats ranged in weight from 32 to 47 g and were distributed to give groups of 5 rats of each sex with approximately the same average weight. Each group was housed with shavings as bedding, and with free access to food and water.

Results. DMA. Fifty-six mice injected intraperitoneally with DMA dissolved in aqueous propylene glycol received doses ranging from 25 to 1000 mg. Propylene glycol concentration varied from 5 to 50% to permit injection of approximately the same volume of solution. These concentrations of glycol produced no reactions in 4 control mice. All mice given 200 mg or less of DMA survived. All those given 400 mg or more died. Four of 7 mice receiving 300 mg died on the second day, indicating an LD_{50} of approximately 300 mg DMA injected intraperitoneally. Death occurred in 2 to 3 hours after doses of 400 and 500 mg, and in about one hour after doses of 600, 800, and 1000 mg. In all instances where death occurred depression developed shortly after injection of DMA, followed by coma.

Mice injected with 25 and 50 mg of DMA differed from controls in exhibiting sensitivity to touch on 6th day after injection. They showed none of the symptoms of surviving mice given 100 mg or more. The latter animals showed increased sensitivity to touch and noise the day after injection. After 24 to 36 hours all survivors periodically held the head to one side, frequently with nodding movements, and in some instances generalized tremors were present. The choreic head movements were frequently followed by circular running. These symptoms persisted during 3 months of observation.

Groups of 4, 4, and 5 mice were given orally 200, 300, and 400 mg respectively of DMA dissolved in 25% aqueous solution of propylene glycol. No deaths occurred, and all animals exhibited the aforementioned symptoms during one month of observation.

None of the mice given daily oral doses of 25 or 50 mg DMA for 3 weeks showed any symptoms until the 8th administration, when some mice on the high dose exhibited circular running movements. At the end of experiment when a total of 16 doses had been given no symptoms had developed on the 25 mg dose, but 2 of the 5 animals receiving 50 mg per dose showed some whirling movements.

Nine rats divided into 3 equal groups were given 100, 200, and 300 mg DMA intraperitoneally. One rat on the highest dose died in 24 hours. All survivors showed the same typical symptoms during 6 months of observation.

ADMA. This reductone, administered orally in 20% gum acacia, was given to 53 mice in doses ranging from 200 to 1500 mg. No deaths and no symptoms occurred in 4 controls given gum acacia, nor in 8 mice receiving 200 mg. Five of 10 mice given 300 mg and 4 of 8 given 400 mg died in 1 to 2 hours. On dosage levels of 500 and 800 mg 25% survival occurred, but mortality was 100% on the higher dosage levels. Convulsions occurred before death, and the heart stopped in systole. No symptoms were noted in any of the survivors.

PIP. Thirty-four mice received this reductone orally as a 20% gum acacia suspension in doses ranging from 400 to 1200 mg. No LD_{50} was established. One mouse receiving 600 mg and 1 receiving 800 mg died after 9 and 6 weeks respectively. Twenty-four hours after administration of all dosage levels spasmodic jerk of the head, generalized tremors and whirling occurred. When observations were discontinued 10 weeks later 25% of the mice given 800 mg or less and 50% of those receiving 1000 or 1200 mg continued to show symptoms.

APIP. A suspension of this reductone in 20% gum acacia was given orally to 46 mice in doses of 200 to 1200 mg. No deaths occurred in controls nor in animals given 200 or 300 mg. Mortality was 25% on doses of 400, 500, and 700 mg; 20% on 800 mg; 75% on 1000 mg; and 100% on 1200 mg. Time of death was unrelated to dosage and varied from 5 to 90 minutes, except on 1200 mg where 2 deaths occurred the following day. No typical symptoms were noted in the survivors.

MORPH. An aqueous 33% propylene glycol solution of MORPH was given intraperitoneally to 61 mice in doses from 100 to 1000 mg and orally to 23 mice in doses from 700 to 1200 mg. No deaths occurred in controls nor in mice injected with 600 mg or less. Mortality was 12.5% after 700 mg, 37.5% after 800 mg, 50% after 850 and 900 mg, and 100% after 1000 mg. No deaths resulted from oral administration of MORPH. Neither intraperitoneal nor oral administration of MORPH produced symptoms characteristic of DMA.

Feeding test-rats—100 days. The two noteworthy effects of feeding the various dietary levels of the 5 reductones were the presence or absence of reductone toxicity symptoms and time of onset, and effects on growth. The typical symptoms of hyperexcitability, elevation and nodding of the head, and whirling were produced only by DMA and PIP.

On 4th day of feeding, rats on a dietary level of 0.06% DMA showed increased activity as compared with controls on the basal diet. At this time daily ingestion of DMA was 55 mg per day. By the 14th day sporadic whirling was seen in some animals and rate of ingestion of reductone was 74 mg. On the 59th day all rats showed whirling. There was no difference in response of male and female rats to the compound. On diet levels of 0.12% and higher typical symptoms were noted on the 4th day and maximum intensity of response to DMA appeared on the 17th to 28th day, whereas maximum response on the 0.06% dietary level occurred about the 59th day. After development of maximum response there was no marked difference between effects on 0.06% and 1.0% dietary levels of DMA.

Increased activity and slight whirling produced by feeding PIP was first noted on the 26th and 3rd day on dietary levels of 0.06% and 0.12% respectively. By the 58th day typical symptoms were developed fully on all dietary levels. Again there was no difference in response of the sexes.

Growth rate was retarded significantly in both male and female rats on all dosage levels of all reductones with the exception of MORPH. Inhibition of growth increased with increasing dietary levels of the reductones. However, only APIP at dietary levels of 0.5 and 1.0% caused a significant decrease in food consumption. In all other instances food intake was equal to or greater than that of the appropriate controls. Increasing toxicity, as judged by effects on growth, places the reductones in the following order: MORPH, DMA, PIP, ADMA, APIP.

Discussion. Cutting and collaborators(7) have described the phenomena produced in mice by dimethylamino-hexose-reductone and compared it with other types of similar activity. The circling movements produced in rats and mice by dimethylamino and piperidino hexose-reductones differ from those produced in rats by magnesium deficiency as described by Kruse, Orent, and McCollum (8). Movement induced by reductones is a rotation in small circles rather than racing in a wide circle, and is not followed by rigidity and death. Symptoms produced by reductones occur in one to 3 days after single doses and persist for long periods of time.

Since long-term feeding tests in rats demonstrated growth inhibition on all dosage levels of all reductones except morpholino, and since anhydrodimethylamino, and anhydro-piperidino-hexose-reductones did not produce whirling under these conditions, it appears that growth inhibition and whirling are unrelated toxic effects.

Summary. The approximate LD_{50} values of dimethylamino-hexose-reductone and morpholino-hexose-reductone injected intraperitoneally in mice were 300 and 850 mg/kg respectively. Oral LD_{50} values of anhydro-

dimethylamino-hexose-reductone, dimethylamino-hexose-reductone, and anhydro-piperidino-hexose-reductone in mice were 300, 400, and 900 mg/kg respectively, while for the morpholino and piperidino-hexose-reductones they were greater than 1200 mg/kg. Single intraperitoneal injections and single oral doses of dimethylamino-hexose-reductone in both mice and rats, as well as long-term feeding of dimethylamino and piperidino-hexose-reductones in rats produced typical symptoms of hyperexcitability, elevation and nodding of the head, and whirling.

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Effect of Colchicine on Taurine Excretion.*† (26434)

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Many of the characteristic biological effects of colchicine parallel those produced by X-rays. Since Kay and Entenman(1) have demonstrated that X-irradiation markedly increases excretion of taurine by the rat, the effect of colchicine on taurine excretion in the rat was studied.

Materials and methods. Male Wistar rats weighing 200-300 g were used, each rat serving as its own control. During the control period urine was collected for at least 2 successive days of 21 hours each, 3 hours of each full control day being allotted to feeding the animals. Food was withheld during the treatment period but water was given *ad libitum* throughout. After the control period the rats were administered colchicine intraperitoneally in doses ranging from 0.8 to 6.4 mg/kg, and urine was collected for various periods up to 36 hours after colchicine administration. Values for taurine output are expressed as a percentage of each animal's taurine output during the control period. For assay of taurine in urine and tissues a modification of the method of Pentz *et al.*(2) was used. In this method extraction of the chloroform-soluble dinitrophenyl (DNP)-derivatives of Dowex-50-treated samples of the urine or tissues leaves DNP-taurine in the aqueous phase. Taurine content is then determined by comparing the absorption of the sample at 355 mμ in a Bausch & Lomb Spectrophotometer with that produced by standard taurine solutions under the same conditions. Paper chromatograms of the DNP-derivatives from urine and tissues in 3 solvent systems showed one component, the mobility of which was the same as authentic DNP-taurine.

Results and discussion. When urine samples were collected over the periods of 0-3, 3-6, 6-14, and 14-36 hours after colchicine administration, it was found that increased taurine excretion begins in the first 3 hours but does not continue beyond 14 hours after treatment. The data also suggest that the peak rate of excretion occurs sometime between 6 and 14 hours following colchicine.

In the control periods preceding treatment taurine excretion averaged 2 micromoles/hr/

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† Preliminary report presented at Fed. Am. Soc. Exp. Biol., April 11-15, 1960, Chicago, Ill., (*Fed. Proc.*, 1960, v19, 42).

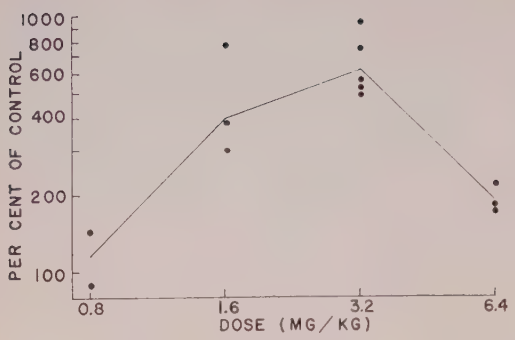

FIG. 1. Taurine output in first 14 hr after various doses of colchicine. Each point represents 1 animal.

rat, or 5.25 mg/21 hr/rat. Rats given 0.8 mg/kg did not excrete increased amounts of taurine but those given 1.6 and 3.2 mg/kg colchicine excreted approximately 4 and 6 times as much taurine, respectively, as they did during their control period(Fig. 1). However, in animals given 6.4 mg/kg colchicine taurine excretion increased to only twice the control values, indicating that this dose was less effective than the 2 lower doses of 1.6 and 3.2 mg/kg. In this respect colchicine differs from X-rays, since Kay, Early and Entenman(3) reported that X-irradiation produced equal and maximal effects in all doses from 250 to 25,000 r. The relative ineffectiveness of such a high dose of colchicine (6.4 mg/kg) in promoting taurine excretion is not readily attributed to kidney failure, since taurine blood levels did not increase significantly 4 hours after either 1.6, 3.2, 6.4 and 12.8 mg/kg colchicine.

Several substances of physiological interest which may be liberated following X-irradiation were investigated to determine whether they might increase taurine excretion in rats. Intramuscular administration of 5 I.U. of vasopressin tannate in oil did not increase urinary taurine excretion significantly. Beta-aminoisobutyric acid and beta-alanine, 2 beta amino acids reported to increase taurine excretion in mice(4), appeared to be relatively ineffective when given intraperitoneally in doses as high as 40 millimoles/kg.

The question as to whether the extra urinary taurine comes from preformed tissue taurine was investigated by determining taurine levels in heart, small intestine and brain at 3, 6, 12, and 24 hours after administration of 1.6 mg/kg of colchicine, a dose which increases taurine excretion about 4 times that of controls. At none of these time intervals was there a significant change in taurine levels nor did significant changes occur in these tissues when they were investigated 24 hours after administering 1.6, 3.2, and 6.4 mg/kg of colchicine. In a separate experiment taurine levels in skin were determined 4 hours after 3.2 mg/kg of colchicine. Taurine in the skin of the paws (618 μg/g), abdomen (460 μg/g) and dorsum (478 μg/g), on the basis of wet weight of the skin, did not change significantly from control levels. Thus, preformed tissue taurine does not appear to be the source of the extra taurine excreted by rats following colchicine administration. Stern and Stim(5) conclude that preformed tissue taurine was probably not the source of the extra urinary taurine excreted after X-irradiation, since taurine levels in muscle, spleen, thymus and liver did not change 24 hours after a dose of 600 r.

Summary. Colchicine in doses of 1.6, 3.2 and 6.4 mg/kg increases urinary taurine excretion in rats, but doses of 6.4 mg/kg are less effective than the two lower doses. Preformed tissue taurine does not appear to be the source of the extra urinary taurine excreted after colchicine administration.

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Lung Localizing Antibodies in Anti-Lung and Anti-Kidney Serum.*† (26435)

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Rabbit antibodies formed against rat lung have been shown to localize in the lungs when injected into rats. There are antibodies also in this serum which localize in other tissues such as kidney(1,2). In this aspect anti-lung serum is similar to anti-kidney serum and also shows nephrotoxic activity(3,4). Moreover, the kidney localizing or nephrotoxic components in anti-kidney serum can be removed by treatment with lung homogenate(4,5). Previous studies of anti-kidney serum by means of radiolabel technic without specific purification have generally failed to show any significant localization in lungs (6). This was due to technical difficulties involving a rather high and variable non-specific uptake of normal serum globulins in the lungs. However, the presence of such lung localizing components in anti-kidney serum, although in minor amounts, has been evidenced by *in vivo* and *in vitro* purification methods(7,8).

The present work utilizing the fluorescein-labeled antibody technic has revealed clearly the presence of lung localizing components in anti-kidney serum as well as in anti-lung serum. Although its content was significantly lower in anti-kidney serum than in anti-lung serum, the lung localizing antibody from both sera seemed to be fixed in the same regions of the lungs.

Experimental. Antisera. Anti-rat kidney sera (referred to as anti-RK) were pooled from rabbits injected with rat kidney homogenate as described previously(9). The saline insoluble fraction of rat lung homogenate sedimented between 1,500 and 25,000 RCF (15 min centrifugation) was used as injecting antigen for anti-lung serum. Anti-lung serum from a single rabbit with a high lung localizing activity was used (anti-RLuH). Normal rabbit serum was a commercial product (Pentex, Inc., Kankakee, Ill.)

The γ-globulin fractions of anti-lung and normal serum were obtained by ethanol fractionation(10) and are referred to as γ G anti-RLuH and γ GNS respectively. Globulin from the anti-RK serum was prepared by ammonium sulfate precipitation (G anti-RK) (11). Radioiodination of proteins was carried out as described previously(12,13). *Detection of localized globulin in the tissues.* Sprague-Dawley rats were used throughout this study. Since the globulins from anti-tissue serum were found to be quite toxic to rats,‡ globulin solutions with or without prior radioiodination were divided into 2 equal portions when 10 mg was administered, and into 3 portions when 30 mg was given (only G anti-RK and γ GNS were used at the higher level). Each portion was injected intravenously at 2 hour intervals. The rats were perfused 18 hours after last injection. In those animals receiving radioiodinated globulins, the tissues were excised and radioactivity was determined as described previously (13). For the fluorescent antibody studies, 6% gelatin-saline solution (C. B. Knox Corp., Camden, N. J.) was infused into lungs *via* trachea, and bronchi of both lobes were tied with silk thread to prevent outflow of gelatin solution. This procedure was used to prevent the collapse of alveolar sacs during the manipulation. The lungs and the kidneys were frozen in dry ice immediately after excision and 4 μ thick sections were cut and stained for the localized globulin with fluorescein-labeled horse anti-rabbit globulin antibody as described previously (14). Rat liver sediment was used instead of beef liver powder to remove non-specifi-

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† Part of the work was presented at 44th Annual Meeting, Am. Assn. Immunol., April, 1960, Chicago, Ill.

‡ 10 mg γ G anti-RLuH and 20 mg G anti-RK were lethal for rats weighing 140-150 g when injected at one time, but 30 mg γ GNS showed no toxic effect.

TABLE I. Amount of Globulin Localized in Tissues.*

I^{131}-globulin inj.	Inj. dose, mg†	% of inj. dose localized (whole organ)					Amt (μg) of globulin localized (whole organ)			
		Lung	Kidney	Liver	Spleen	Blood‡	Lung	Kidney	Liver	Spleen
γ G anti-RLuH	10	.81	.26	1.00	.11	38	81	26	100	11
G anti-RK	30	.28	.74	1.08	.16	19	84	222	324	48
γ GNS	30	.44	.08	.29	.03	40	132	24	87	9

* Four rats were used for each group. Rats were perfused 18 hr after last inj. and radioactivity of the tissues determined.

† γ G anti-RLuH was inj. in 2 equal portions and γ G anti-RK and γ GNS were inj. in 3 equal portions at 2 hr intervals.

‡ Calculated assuming total blood as 1/10 of body wt.

cally staining components in labeled horse antibody preparations.

Results and discussion. Preliminary results indicated that localization of radioactivity in the lungs of animals one day after injection with G anti-RK or γ GNS was less than ½ the localization found in animals receiving γ G anti-RLuH (on the basis of percent of injected dose localized in the tissue). Therefore, pairs of animals were injected with γ-globulin fractions of anti-lung serum (10 mg), anti-kidney serum (10 mg and 30 mg), or normal serum (10 mg and 30 mg). The rats were killed after one day and observed for localized globulin by the fluorescein technic as described above. At the 10 mg dose level, only animals receiving γ G anti-RLuH showed positive staining in the lung. However, when the dose was increased to 30 mg, the group receiving G anti-RK became positive whereas those receiving γ GNS remained negative even at this level. Positive staining was seen in the kidney with both γ G anti-RLuH (10 mg) and G anti-RK (10 mg and 30 mg), but not with γ GNS (10 and 30 mg).

Total amounts of globulin localized in the lung were estimated by injecting the same preparations with radioiodine. Amounts of 10 mg of γ G anti-RLuH, 30 mg of G anti-RK or γ GNS were injected into 4 rats for each group (Table I). The weight of globulin localized in the lungs was approximately the same for animals receiving the anti-lung and the larger amount of anti-kidney preparations. For animals receiving 30 mg of the normal serum preparation, the weight localized was even greater.

The negative results obtained by fluorescein label technic with even 30 mg of γ GNS indicate that it is rather uniformly distributed throughout the lung tissue (or washed out during the staining procedure) whereas the lung localizing antibody in anti-RLuH and in anti-RK must be fixed tightly at local concentrations high enough to give staining (15). Indeed in the assay of globulin preparations directly for lung localizing antibodies the use of the fluorescein label technic is especially effective. The background fixation of protein from radiolabeled γ GNS is variable from sample to sample (0.3-0.7% of injected dose). Actually, the observed localization from G anti-RK falls within this range and only with the fluorescein technic is a lung localizing activity, not present in γ GNS, observed without resorting to specific purification procedures. (Radioautographs might also show the high local concentration.)

Radiolabel technics with specific purification do show lung localizing antibodies in G anti-RK(7,8) and do not require administration of the high levels of protein required with the fluorescent method(15).

The positive staining of the lung sections is primarily localized in certain areas of alveolar walls excluding nuclei (Fig. 1). Arteries, veins, bronchi and bronchioli showed no staining. Although the fragility of lung tissue in frozen state prevented us from identifying the exact location of localized globulin in finer detail, both endothelial and epithelial cells seemed to be stained. No difference in staining pattern was observed between anti-RLuH and anti-RK.

FIG. 1. Section of lung tissue of rat inj. with 10 mg γ G anti-RLuH and stained with fluorescein labeled horse anti-rabbit globulin antibody. Although staining is confined to the alveoli, the difference in intensity in certain regions of the alveoli indicates a differential concentration of γ G anti-RLuH at these sites (see figure). Nuclei were not stained.

It is interesting that the lung localizing component in both anti-lung and anti-kidney serum seems to be fixed in the same region. This does not necessarily mean that they are reacting with the same antigen, but it implies that the lung antigens responsible for cross-localization of anti-kidney antibody seem to be present in the same region as those responsible for fixation of anti-lung antibody. Indeed, that the responsible antigens may be different for these 2 types of lung localizing components has been suggested by previous studies on the *in vivo* purification of anti-kidney and anti-lung antibody(7). The radioglobulin recovered from the lungs of animals receiving anti-lung antibody, when reinjected, localized in the lung tissue to a high extent, but not in the kidney. In contrast, the similar preparation from the lungs of animals receiving anti-kidney antibody, localized in the kidney as well as in the lung, indicating the presence in the lung of antibodies capable of localizing in kidney.

Summary. Localization of antibodies in lungs was examined by fluorescent antibody technic after injection of rabbit anti-rat lung and anti-rat kidney serum into rats. Although both sera gave positive staining in lungs, anti-lung serum contained significantly higher amount of lung localizing antibodies than anti-kidney serum. The localizing components in both sera seemed to be fixed in the same region of alveolar walls.

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Effects of Vitamin D and Cortisone on Intestinal Absorption of Calcium in the Rat. (26436)

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The importance of Vit. D in promoting intestinal absorption of calcium has long been recognized(1,2), but its mechanism of action remains unknown. The work of several investigators(1-4) strongly suggests that large doses of Vit. D can lead to excessive calcium absorption. However, other studies(1,2,5,6) indicate that calcium mobilization from bone is the predominant cause of the hypercalcemia of Vit. D overdosage. The hypercalcemia can be corrected by cortisone administration(7,8). Though this effect has been attributed(8,9), at least partially, to a decrease in intestinal calcium absorption, the mode of cortisone action in this situation has not been clearly defined.

The present study was designed to determine 1) whether large doses of Vit. D will increase intestinal calcium absorption above that induced by physiologic amounts, 2) whether cortisone will inhibit calcium absorption in presence of either physiologic or excessive amounts of Vit. D, 3) whether different areas of the intestine differ in ability to absorb calcium physiologically, and whether additional Vit. D and cortisone may affect absorption differently in various intestinal areas.

To eliminate any bone or kidney effects on calcium metabolism, segments of small intestine were studied *in vitro,* using a modification of the gut sac technic of Wilson and Wiseman(10). Similar technics have recently been used by others (11-14) for studying intestinal transport of calcium.

Materials and methods. Male Sprague-Dawley rats weighing 100-130 g were treated with daily doses of 20,000 units of Vit. D* by stomach tube, or 2.5 mg cortisone acetate† subcutaneously, or a combination of both drugs. A control group received cottonseed oil and saline, which were the vehicles for the Vit. D and cortisone respectively. During treatment, the rats were allowed *ad lib.* tap water and a nutritionally complete diet containing 1.68% calcium, 0.8% phosphorus and 148 units of Vit. D per 100 g of diet. On the 6th day of this regimen the rats were killed by decapitation. A 3 cm segment of small intestine was quickly removed from 3 different areas (duodenum, distal jejunum, distal ileum) and everted. A sac was prepared from each segment by tying both ends, after placing in the lumen 0.35 ml of a solution of the following composition: 0.148 M NaCl, 0.008 M sodium phosphate of pH 7.4, 0.02 M glucose, 4×10^{-5} M $CaCl_2$, and 0.02 μc/ml $Ca^{45}Cl_2$ (Oak Ridge Nat. Labs, specific activity $>$ 1000 mc/g). The filled sac was placed in an Erlenmeyer flask containing 2.5 ml of solution identical to that placed in the lumen. Because the mucosal surface was now on the outside and the serosal surface on the inside, any material moving from mucosal to serosal surface was collected inside the gut sac. The flask was aerated for 1 minute with oxygen, stoppered, and placed in a shaking incubator for 2 hours at 37°C. The sac was then removed from the bath, blotted of adherent outside solution, and the contents drained into a small tube. (Volume of fluid recovered in this manner was approximately 85% of that used to fill the sac for the duodenal segments and 95-105% for the jejunal and ileal segments.) Post-incubation Ca^{45} concentration was determined on equal aliquots of inside (serosal) and outside (mucosal) solution by drying on planchets and counting with a thin end window Geiger-

* Kindly furnished by Brewer and Co., Inc., Worcester, Mass.

† Kindly furnished by Sharpe and Dohme, Division of Merck and Co., Inc., Philadelphia, Pa.

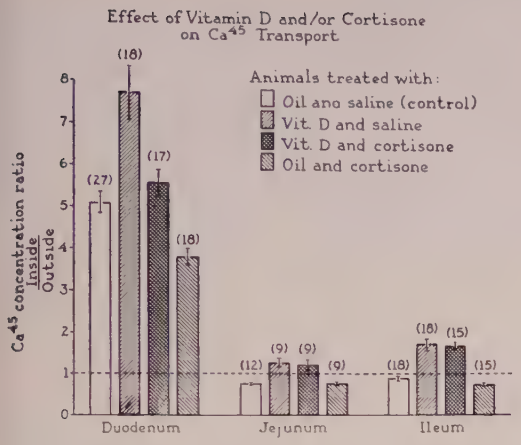

FIG. 1. Effects of vitamin D and/or cortisone on Ca^{45} transport. Dotted line indicates the I/O ratio existing in all segments before incubation. Each bar represents mean post-incubation value, with stand. error, for number of animals studied (indicated in parentheses).

Mueller tube. From these determinations, the Ca^{45} concentration ratio inside solution/outside solution (I/O ratio) was calculated. Since by design, the Ca^{45} I/O ratio was 1 before incubation, this ratio after incubation was used as a measure of transport of Ca^{45} through the intestinal wall during incubation period.

Results. Calcium transport was much greater in the duodenum than in the other 2 intestinal areas (Fig. 1). In the duodenal segment, the I/O ratio was 5.0 for control rats and 7.7 for Vit. D-treated rats, indicating a statistically significant increase ($P < 0.001$) in calcium transport. Cortisone significantly decreased calcium transport of Vit. D-treated rats to 5.6, and of non-Vit. D-treated rats to 3.8 ($P < 0.001$ and $P < 0.01$ respectively). In the jejunal and ileal segments, there was no calcium transport in control rats, but significant transport ($P < 0.001$) in Vit. D-treated rats. Cortisone did not significantly depress the Vit. D-induced transport of these 2 segments.

Discussion. Since the present study demonstrates movement of calcium through the wall of the gut sac against a concentration gradient, and aerobic conditions are required by this experimental system(11), the observations indicate active calcium transport. This active transport of calcium was not uniform throughout the length of the small intestine. In the presence of physiologic doses of Vit. D, active transport was demonstrable in the duodenal segment only. (The I/O ratio of slightly less than 1 in jejunal and ileal segments of the control rats (Fig. 1) would suggest transport of calcium in the opposite direction. However, the slight dilution of Ca^{45} due to 1) mixing with tissue stable calcium and 2) the slight water absorption in these segments is considered a more likely explanation for the values being slightly less than 1). Large doses of Vit. D increased active calcium transport in the duodenal segment, and initiated active transport in jejunal and ileal segments. Cortisone reduced calcium transport in the duodenum, in the presence of either physiologic or excessive amounts of Vit. D. In contrast, cortisone did not reduce Vit. D-induced calcium transport in the jejunum and ileum.

The present observations do not indicate whether the variation in response of different areas of the intestine to Vit. D and cortisone is due to differences in tissue responsiveness, or to dissimilar mechanisms of action of these substances on different areas of the gut. The mechanism by which cortisone has an effect on calcium transport which is opposite to that of Vit. D is not defined. However, the variable relationship between these opposite effects in the different intestinal segments suggests that competitive inhibition is not an adequate explanation.

The importance of active transport for calcium absorption *in vivo* is not clear. Though calcium concentration of the solution used in the present study was much less than physiological, additional observations by us, and by others(11), indicate that active transport is demonstrable against a calcium concentration equal to that of normal plasma. Therefore, active transport of calcium *in vivo* is at least possible. In the normal intact animal, where calcium concentration of the intestinal contents is generally greater than that of extracellular fluid, absorption could occur by passive diffusion, but other mechanisms may well be involved. The authors suggest that active transport may at

least become an important mechanism when dietary calcium is limited and therefore an adequate gradient for calcium absorption by diffusion may not exist.

Summary. Using an *in vitro* gut sac technic, active transport of calcium through the intestinal wall of the rat has been demonstrated. In the presence of physiologic amounts of Vit. D, active calcium transport is confined to the upper portion of the small intestine. In this area, large amounts of Vit. D increase, and cortisone decreases, calcium transport. In the jejunal and ileal areas, active transport occurs only when large amounts of Vit. D are present, and this transport is not reduced by cortisone.

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Effect of Hydrocortisone on Herpes Virus-Infected HeLa Cells.* (26437)

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Certain of the effects of steroids on cell metabolism and growth of both normal and virus-infected cells have been studied in tissue culture systems(1-7), and the magnitude of effect of steroid on cellular growth has been found to be related to the origin of the cells. Holden and Adams(1) and Grossfeld (2) found a 40-50% reduction of growth rate of fibroblastic cells in tissue culture in presence of hydrocortisone, whereas Grossfeld and Ragan(3) found that similar treatment of cells of epithelial origin had no effect. Franklin and Sinclair(4) found similar inhibition of growth of fibroblastic cells but reported growth stimulation of epithelioid cells treated with prednisolone. The mechanism of growth inhibition of fibroblastic cells would appear to be associated with cytotoxic effects of steroid since Kline *et al.*(5) found that cell lines of connective tissue origin were much more susceptible to the cytotoxic effects of steroid than were cell lines of epithelial origin.

The effect of steroid on cell metabolism has been studied by Grossfeld(6) who found that hydrocortisone increased rate of glycolysis and O_2 uptake of mouse fibroblast cells (strain L). Similarly Fisher and Fisher(7) found that HeLa cells treated with cortisone had a greater rate of glycolysis than did control cells, although control cells utilized slightly more glucose than did the cortisone-treated. Infection of these cells(7) with herpes virus resulted in an increase in rate of

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glycolysis of both steroid-treated and untreated cells as well as a significant rise in glucose used, and total acids produced by infected cells treated with cortisone over the values obtained with virus or steroid alone. That study indicated that virus and cortisone together produced qualitative and quantitative changes in cellular metabolism not found when either substance was added singly.

Experiments reported here were carried out to determine whether herpes virus infection influences uptake of hydrocortisone by HeLa cells and the effect of steroid and virus on glucose utilization by cells.

Methods. Virus. HF strain of herpes simplex virus grown in HeLa cell cultures and stored at –60°C was used as virus stock. Titer of this stock virus used in all experiments was about 10^6 pock forming units (PFU)/ml as determined by titrations on the chorioallantoic membrane of 11-day embryonated hens' eggs.

Tissue culture. HeLa cell cultures were grown as monolayers in tubes using a medium (N_{16}) described by Puck(8), supplemented with 30% fetal calf serum. At the start of experiments, glucose and serum concentrations were reduced to the levels indicated.

Hydrocortisone. Hydrocortisone (Solu-Cortef, Upjohn Co.) and C^{14}-4-hydrocortisone with a specific activity of 42.4 μc/mg (Tracerlab, Waltham, Mass.) were used. Labelled steroid was diluted with unlabelled hydrocortisone to give a final concentration of 200 μg of hydrocortisone/ml in the tissue culture medium, and concentrations of labelled hydrocortisone of 1% in the first experiment and 5% in the second. Isotope counts were made on a gas flow analyser.

Glucose. Determinations of glucose concentrations were made using the anthrone method described by Seifter *et al.*(9)

Results. Monolayer cultures of HeLa cells were washed and the medium replaced by one containing 5% fetal calf serum and 600 μg glucose/ml. Virus was added to the cultures in a concentration which resulted in a ratio of 1 PFU of virus for each 16-20 cells. The amount of glucose present in the virus inoculum was sufficiently low as not to add a detectable amount to that present in the medium. Labelled and unlabelled hydrocortisone were added to the cultures in the amounts previously indicated at the same time as virus inoculum. Cultures were incubated at 37°C and 4°C. After 40 hours' incubation at 37°C, virus-infected cells showed initial cytopathic changes on microscopic examination as evidenced by an increase in rounding and granularity of cells; by 96 hours cultures were almost totally destroyed. At the end of 40 hours' incubation, tissue culture fluids were removed from the majority of cultures for determinations of glucose. Hydrocortisone concentrations were measured by determining the amount of C^{14}-labelled steroid present. Similar determinations were made on the cells which were collected by trypsinization, washed, and counted in a hemocytometer. The amounts of steroid found in the tissue culture medium and with the cells accounted for the total amount of hydrocortisone added. The results of these experiments are shown in Table I, where each determination represents the mean of 5 separate samples, and each sample is a pool of 3 cultures. The amount of hydrocortisone associated with the cells was similar in both experiments, and virus infection did not influence the amount of steroid taken up. The amount of hydrocortisone found with the cells represents about 10% of total amount available based on calculations of uptake per 10^6 cells. This figure correlates well with similar determinations of hydrocortisone remaining in the medium after 40 hours' incubation. These results suggest that stress of virus infection does not influence uptake of steroid by cells in tissue culture. Similarly, results obtained with cultures incubated at 4°C were almost identical to those at 37°C indicating that uptake of hydrocortisone by cells is independent of temperature.

The amount of glucose taken up by cells over a 40-hour incubation period was calculated by subtracting the amount present at 40 hours from that present in fresh medium. When glucose uptake is calculated in terms of a unit cell population rather than in total culture, the increase in glucose uptake in presence of hydrocortisone is changed to a

TABLE I. Hydrocortisone and Sugar Uptake by HeLa Cells in Presence and Absence of Herpes Virus.

Exp.	Virus	Hydrocortisone	Hydrocortisone uptake/10^6 cells at 37°C	Calculated glucose uptake		Cell conc. of glucose/10^6 cells
				Total culture	Per 10^6 cells	
				μg		
1	—	—		261	155	7.0
	—	+	12	297	145	2.8
	+	—		208	107	6.1
	+	+	15	258	120	3.7
2	—	—		382	187	6.2
	—	+	17	378	170	2.6
	+	—		308	134	5.7
	+	+	15	358	150	3.9

decrease because of greater numbers of cells present in these cultures (Table I). It is not known whether this increase in cells in steroid-treated cultures is the result of increased growth or a better survival of cells under conditions of reduced nutrients in the medium (5% serum and reduced glucose). The possibility of growth stimulation in presence of hydrocortisone is supported by the work of Franklin and Sinclair(4). Addition of virus significantly reduced the uptake of glucose; however, in the presence of hydrocortisone, the effect of virus in reducing glucose uptake was not as great. The glucose content of cells is shown in the last column of Table I. Cellular levels of glucose are decreased by addition of hydrocortisone and left virtually unchanged by infection with virus, suggesting that cellular metabolism of glucose is changed in the presence of steroid while uptake is only slightly affected. The differences in cellular levels of glucose between steroid-treated and control cells and between steroid-treated cells infected and not infected with virus are significant to 1% and 10% levels respectively.

Discussion. The finding that hydrocortisone was associated in equal amounts with infected and noninfected cells at 37°C and 4°C indicates that the union of steroid and cell is nonspecific in these epithelial cells. However, it may be that epithelial and fibroblastic cells react differently in this regard as they do in others(1-5).

The results of studies on glucose uptake by herpes-infected HeLa cells are in partial disagreement with those of Fisher and Fisher (7) in that steroid and virus did not increase uptake of glucose above that of control cultures. This difference in results may be due to the larger virus inoculum (about 50 times greater in terms of PFU/cell) used in the present study resulting in a greater physiological impairment of cells by 40 hours, which was the same time after infection that Fisher and Fisher(7) made their observations. The decrease in amounts of glucose in cells treated with hydrocortisone tends to support the observation of Fisher and Fisher (10) that alternate pathways of glucose metabolism are operative in steroid-treated cells which in the present case has resulted in either a greater utilization of endogenous reserves or a shift away from processes leading to glucose storage. Effects of hydrocortisone on herpes-infected HeLa cells seem to result in a partial return of cells to a normal state in terms of glucose uptake and storage.

Summary. Association of C^{14}-labelled hydrocortisone with herpes virus-infected HeLa cells appeared nonspecific in that equal amounts of steroid were taken up at both 37°C and 4°C by noninfected as well as infected cells. The action of steroid on the cells resulted in a slight decrease in glucose uptake and a proportionally greater decrease in glucose stored. When steroid-treated cells were infected with virus, there was an increased uptake of glucose over that of virus-infected controls, and more glucose stored than in control cells treated with hydrocortisone suggesting that the action of steroid results in a partial return of stressed cells to more normal physiologic function.

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Effect of Antiserum on Adsorption of Vaccinia Virus to Earle's L Cells.* (26438)

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Neutralized virus is, by definition, not measurable by conventional titration methods. For this reason it has been difficult to assess the part played by altered host cell-virus particle adsorption in the overall neutralization of virus by antiserum. Recent work(1) with Newcastle disease virus, carrying radioactive label, has shown a blocking of adsorption of neutralized virus to host cells. A more direct demonstration of this vital step in the infectious sequence is desirable.

We have described(2) the methods for counting virus particles released by sonic energy from host cells shortly after adsorption. This paper contains preliminary results of application of these methods which show that a substantial part of the neutralization of the infectivity of the virus for L cells is manifest at the point of adsorption.

Materials and methods. Vaccinia virus, WR (mouse neurotropic) strain was obtained from the American Type Culture Collection, passed 18 times in L cells and stored at –70°C. L cells were obtained from Dr. W. Earle's laboratory. Medium and other details of the cell cultures have been described(3). Antiserum was produced by vaccination of young New Zealand white rabbits with active virus. Further applications of live virus were made 5 and 12 weeks later with the last application resulting in a typical immune response. Blood was drawn at the 13th week and serum was stored at –20°C. Normal serum was obtained by cardiac puncture from rabbits before vaccination. All sera were heated 30 minutes at 56°C before use. Counting of virus particles was done by the agar sedimentation process (4) employing the electron microscope.

Experimental. Mixtures of active virus and serum were made in growth medium such that they contained 10^9 virus particles per ml and a serum dilution of 1/5. These were incubated 4 hours at 37°C with occasional agitation. L cells were counted, sedimented in pointed centrifuge tubes and resuspended in the various serum-virus mixtures in such quantity that final concentration was 5×10^5 per ml. There were thus 200 virus particles per cell. Adsorption proceeded at 37°C with frequent agitation for 1½ hours, after which the cells were washed twice and the remaining adsorbed virus released by 2 minutes treatment with 9 KC sonic energy. This cell-

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TABLE I. Effect of Antiserum in Reducing Adsorption of Vaccinia Virus to L Cells.

Serum in medium	L cells/ml suspension	Virus particles released/ml of sonic treated, washed cells	Virus particles/cell
Normal rabbit	3.0×10^5	3.6×10^6	12
Immune "	3.7×10^5	$<3.6 \times 10^5$	<1

associated virus was then counted.

Results of a typical experiment are shown in Table I. An average of 12 virus particles was found per L cell when adsorption of active virus proceeded in presence of normal serum, while none were found when neutralized virus was used in presence of antiserum. This encouraging result led to further experiments where adsorption could be increased and the results expressed in larger and, therefore, more statistically significant numbers.

Sedimentation adsorption experiments were made with the same serum-virus-cell mixtures as those above. Immediately after addition of cells the mixtures were sedimented at 8000 G for 7 minutes, depositing both cells and virus in a thin layer on a smooth agar receiving surface(5). Cell density on this surface was 5×10^5 per cm^2.

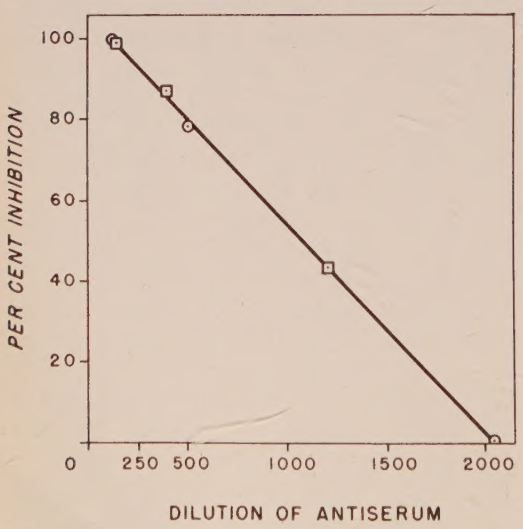

FIG. 1. Neutralization of vaccinia virus by rabbit antiserum as measured by plaques on L cell monolayers. % inhibition is:

$$100\left(1-\frac{\text{No. of plaques with antiserum dilution}}{\text{No. of plaques with normal serum dil.}}\right).$$

After resuspension, washing and sonic treatment of the cells, the adsorbed virus was released by sonic treatment and the particles counted. Of the 200 virus particles available per L cell, an average of 70 was found adsorbed in presence of normal rabbit serum and 60 with normal horse serum. With immune rabbit serum, only 14 particles were found per cell (Table II). These data are typical of this reaction, showing that 80% of the virus adsorption which proceeds in normal rabbit serum is prevented by the immune serum of a dilution of 1-5. The small difference in results with normal horse and rabbit sera is probably not significant.

TABLE II. Effect of Antiserum in Reducing Adsorption of Virus to Cells Even When Contact Is Increased by Centrifugal Force.

Serum in medium	L cells/ml suspension	Virus particles released/ml of sonic treated, washed cells	Virus particles/cell
Normal rabbit	4.7×10^5	3.3×10^7	70
" horse	5.5×10^5	3.3×10^7	60
Immune rabbit	4.7×10^5	6.8×10^6	14

Evaluation of the antiserum was made by titrating residual virus activity on monolayers of L cells. Equal amounts of virus were added to serial 3X dilutions of serum and the mixtures held at 37°C for 30 minutes. A 0.1 ml sample of each dilution was inoculated, in duplicate, onto monolayers in 1 ounce prescription bottles. These bottles were incubated at 37°C for 3 hours with frequent tilting to allow for even distribution. The monolayers were then washed with Hank's BSS and overlaid with 1% Noble agar in nutrient fluid. On the third day a second overlay containing neutral red (1: 50,000) was added. Controls consisted of untreated virus and virus treated with normal rabbit serum.

Results of 2 separate titrations are plotted in Fig. 1. Cultures receiving immune serum dilution about 1-1000 showed half as many plaques as controls with normal serum.

Discussion. Although much has been written about neutralization of virus by specific antiserum, most of the comment is highly speculative regarding the part played by an-

tibody in the process of adsorption of virus to host cells. Hulten and McKee(6) have suggested the existence of 3 zones of neutralization of influenza virus. In the first of these some virus propagation can still proceed; in the second this is stopped but virus fixation to the cell still occurs; while in the third, "probably" fixation of virus to the cell is also blocked. Rubin and Franklin(1) working with radioactive NDV conclude, somewhat similarly, that although one antibody molecule per virus particle will not block all particle adsorption, it does block infection. They believe several molecules of antibody are necessary to prevent adsorption of the virus particle to the cell.

The data presented here show with singular directness that 4/5 of the expected adsorption of vaccinia virus to susceptible L cells was blocked by antiserum. During these experiments, washing of the cells was sufficient to reduce the amount of unattached virus below the counting level. Virus and cells were brought into close proximity by centrifugal force. In the experiments involving ordinary mixing, no adsorption was observed. It must be remembered, though, that counting of virus particles at the level of one per cell is difficult. This experiment shows only that adsorption is blocked by antibody at least as much and possibly more than in the one employing centrifugal force. Work is continuing to determine whether quantity of antibody required to cause a given amount of adsorption inhibition is determined by total number of virus particles or by number of infectious units.

Summary. Antivirus rabbit serum is shown to block 80% or more of the adsorption of vaccinia virus to susceptible L cells. Data supporting these conclusions are provided by direct virus particle counts with the electron microscope.

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